The author hereby certifies that the use of any copyrighted material in the thesis manuscript entitled:

"Pharmacologic Regulation of Peroxisome Number in Glia"

is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.

Megan Hershfield
Neuroscience Program
Uniformed Services University

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2008		**00-00-2008 to 00-00-2008**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Pharmacological Regulation Of Peroxisome Number In Glia	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University Of The Health Sciences,4301 Jones Bridge Rd,Bethesda,MD,20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**79**	

APPROVAL SHEET

Title of Thesis: "Pharmacological Regulation of Peroxisome Number in Glia"

Name of Candidate: Megan Hershfield
Neuroscience Program
Master of Science
Date

Thesis and Abstract Approved:

Michael Schell, Ph.D. — Date: 9-2-08
Department of Pharmacology
Thesis Advisor

Thomas Cote, Ph.D. — Date: 8-29-08
Department Pharmacology
Committee Member

Aviva Symes, Ph.D. — Date: 9.16.08
Department
Committee Member

ABSTRACT

Title of Thesis: "Pharmacological Regulation of Peroxisome Number in Glia"

Author: Megan Rose Hershfield, Master of Science 2008

Thesis directed by: Michael J. Schell, Assistant Professor
Pharmacology Department, Neuroscience Program

Peroxisomes are sites of specialized biochemical reactions within all cells. The pharmaceutical regulation of peroxisome number has potential as medical therapy, but the mechanisms that control peroxisome number in brain cells are largely unknown. Our data suggest a novel relationship between cellular cholesterol homeostasis and peroxisome number in brain cells. Using primary cultures of rat cerebral cortical astrocytes, we have identified novel means of regulating the cellular density of peroxisomes via the liver X receptor (LXR), a nuclear receptor that controls cholesterol efflux in cells. Our investigation reveals a novel means of pharmaceutically induced peroxisome proliferation in astrocytes and the possibility of new therapies for treating disorders in peroxisome biogenesis and metabolism. We have also provided a new line of evidence to suggest a relationship between cholesterol homeostasis peroxisome abundance that may have relevance to therapies for Alzheimer's disease and Neimann-Pick type C disease.

PHARMACOLOGICAL REGULATION OF PEROXISOME NUMBER IN GLIA

By

Megan Rose Hershfield

Thesis submitted to the Faculty of the

Neuroscience Graduate Program of the

Uniformed Services University of the Health

Sciences in fulfillment of the

requirements for the degree of

Master of Science 2008

DEDICATION

To my Babcia
(1910-2003)

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 1

Chapter 2

Chapter 3

LIST OF ABBREVIATIONS

4-PBA	4-phenylbutyrate
24(S)-OH	24(S)-hydroxycholesterol
AD	Alzheimer's Disease
apoE	apolipoprotein E
CHO	Chinese hamster ovary
CNS	central nervous system
CSF	cerebral spinal fluid
DLP1	dynamin-like protein 1
ER	endoplasmic reticulum
GFP	green fluorescent protein
HDAC	histone deacetylase
HDL	high-density lipoprotein
LXR	liver X receptor
NPC	Niemann Pick type C disease
PBD	peroxisome biogenesis disorder
PTS	peroxisome targeting sequence
PPAR	peroxisome proliferators activated receptor
Pex	peroxin
PXR	pregnane X receptor
RAR	retinoid acid receptor
RXR	retinoid X receptor
SREBP	sterol regulatory element binding protein

T09	T0901317
VLCFA	very long chain fatty acid
X-ALD	X-linked adrenoleukodystrophy
ZS	Zellweger’s syndrome

Chapter 1

All eukaryotic cells possess single-membrane organelles called peroxisomes. Peroxisomes are sites of specialized biochemical reactions, including the -oxidation of long-chain fatty acids, the oxidation of cholesterol to produce bile acids, plasmalogen synthesis, the oxidation of D-amino acids, and the catabolism of hydrogen peroxide. Although peroxisomes are most often studied in the major metabolic organs such as liver or kidney, they also have great relevance in neurobiology. Evidence for this comes from the severe neurological phenotypes of patients with peroxisomal biogenesis disorders (PBD), such as Zellweger's syndrome (ZS) (Gould & Valle 2000). Over 30 peroxisomal proteins, termed peroxins (Pex), have been discovered thus far; deletions of peroxins lead to malfunctions in metabolism, such as an inability to import very long-chain fatty acids into the peroxisome for β-oxidation, a deficit that causes X-linked adrenoleukodystrophy (X-ALD) (Poulos *et al.* 1992, Honsho *et al.* 1998).

The pharmaceutical control of peroxisome number is medically relevant. For example, drugs that cause an increase in peroxisome numbers, termed peroxisome proliferators, have shown promising results in animal models of X-ALD through enhancing the metabolism of long-chain fatty acids (Gondcaille *et al.* 2005, Fourcade *et al.* 2001). The core biochemical mechanisms by which cells control peroxisome number are well studied [for example, see (Fagarasanu et al. 2007)]. Yeast genetics have identified more than 32 proteins that control peroxisome number (van der Zand et al. 2006). Most of the Pex proteins reside on or in the peroxisomal membrane, but a few

occur preferentially either in the cytosol (where they act as chaperones to shuttle other peroxisome components to the organelle), or else in the ER, which is involved in peroxisome membrane biogenesis (see below). All Pex proteins are conserved in higher eukaryotes, and eukaryotes express a few additional Pex proteins that do not occur in yeast. These metazoan-specific Pex proteins are known because humans who harbor mutations present with Zellweger's syndrome, the absence of peroxisomes.

The cellular signals that regulate the actions of the Pex protein—and thereby lead to modifications in the rates of peroxisome proliferation, fission and degradation—are poorly understood. Environmental toxins as well as metabolic intermediates are known to trigger peroxisome proliferation (Chang et al. 1999), but it remains unclear when peroxisome proliferation is desirable versus detrimental. As neurodegenerative disorders such as Alzheimer's disease (AD) and Niemann-Pick type C (NPC) disease have been associated with deficient lipid metabolism and increased accumulation of byproducts, the pharmaceutical targeting of peroxisome biosynthesis, maintenance and degradation has great potential as therapy.

Peroxisomal Synthesis and Degradation

The number of peroxisomes in a cell reflects the balance between synthesis and degradation. Previous studies have indicated two candidate pathways of peroxisome biosynthesis. In the *de novo* model, peroxisomes form via budding from the endoplasmic reticulum (ER) and share a subset of membrane and protein components with the ER (Novikoff & Novikoff 1972). The fission model envisions the growth and division of

preexisting peroxisomes coupled with posttranslational protein import, similar to what occurs for mitochondria (Lazarow & Fujiki 1985) . Compelling evidence indicates that both models occur in cells (Figure 1). Numerous studies report the fission of mature peroxisomes [for example, see (Koch et al. 2004)]. Recently, nascent peroxisomes produced *de novo* have been visualized during their budding from the ER (Kim et al. 2006). Notably, mutations that lead to a complete lack of peroxisomes in cells can be reversed once the wild-type gene is re-introduced (South & Gould 1999). Thus, in many cell types, the *de novo* pathway appears to be the chief generator of the peroxisome population in cells, and the fission pathway secondary.

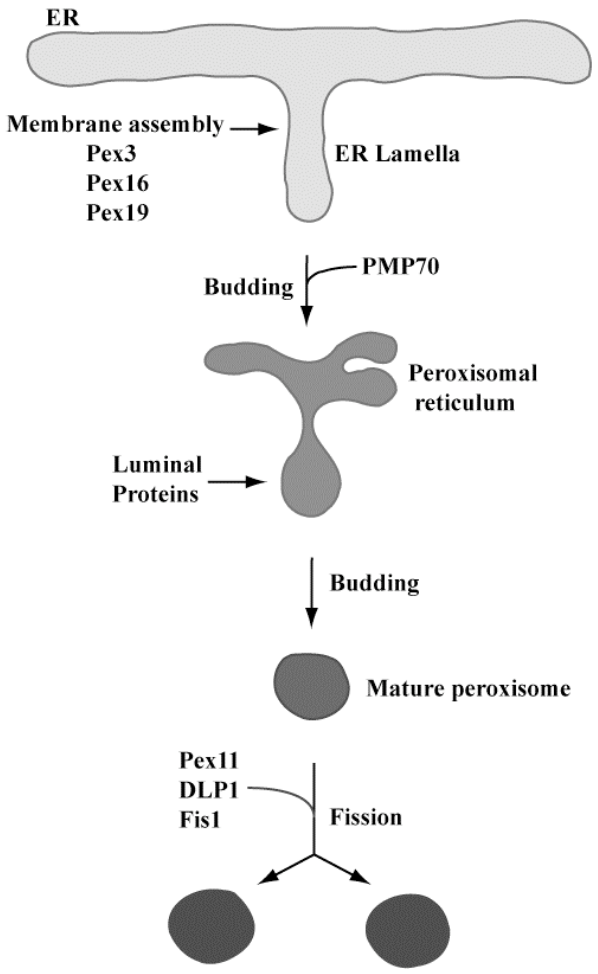


Figure 1. Illustration of the peroxisomal proliferation pathways. Budding off the ER lamellar region and subsequent importation of matrix and membrane proteins is facilitated by Pex3, Pex16 and Pex19. Further maturation occurs via scavenging proteins Pex5 and Pex7. Once mature, fission machinery (Pex11, DPL1 and Fis1) elongates and divides the peroxisome in response to cellular and environmental cues.

De novo synthesis begins in specialized lipid-rich regions of the ER (Boukh-Viner & Titorenko 2006), which contain clusters of peroxisomal proteins that become incorporated into the organelle membrane when budding occurs (Hoepfner et al. 2005). These nascent peroxisomes (sometimes referred to as “pre-peroxisomes” or “ghosts”) have only the bare minimum of proteins required for function. The membrane proteins then act as docking locations for peroxisomal shuttling proteins, Pex5, Pex7 and Pex19. These scavenger-proteins, synthesized on free ribosomes, are located in the cytosol and recognize peroxisomal targeting sequences (PTSs) that are present on proteins in the cytosol destined for peroxisomal import. As peroxisomes mature, enzymes required for proper function are imported. The fission of mature peroxisomes has been explored extensively in recent years following the discovery of multiple proteins that are involved. This process involves the elongation and constriction of the peroxisome along its length through the actions of Pex11β, giving to the description “beads on a string” (Schrader et al. 1998). Through the combined efforts of dynamin-like protein 1 (DLP1) and Fis1, segregation and fission occurs (Kobayashi et al. 2007). This process shares many molecular components with mitochondrial fission.

While the pathways of peroxisome synthesis have been a source of controversy for over 40 years, our understanding of the degradation pathway is much clearer. Termed “pexophagy”, this autophagy-related pathway has been studied intensively in yeast. Upwards of two-dozen proteins are involved in this process, including Pex3p and Pex14p; two peroxins involved in both organelle synthesis and cargo import into the peroxisome (Bellu *et al.* 2001a, Bellu *et al.* 2002). Pexophagy of mature peroxisomes begins with the removal of Pex3p from the peroxisomal membrane (Bellu et al. 2002). Its

deletion allows the docking of degradation factors such as Atg11 to Pex14p (de Vries et al. 2006). Degradation of existing peroxisomes can be accelerated or slowed in response to available nutrients, as seen with yeast grown on different mediums (Bellu *et al.* 2001b, van der Klei *et al.* 1991). Similarly, peroxisomal populations in higher eukaryotes are strongly influenced by metabolic alterations (Chang et al. 1999).

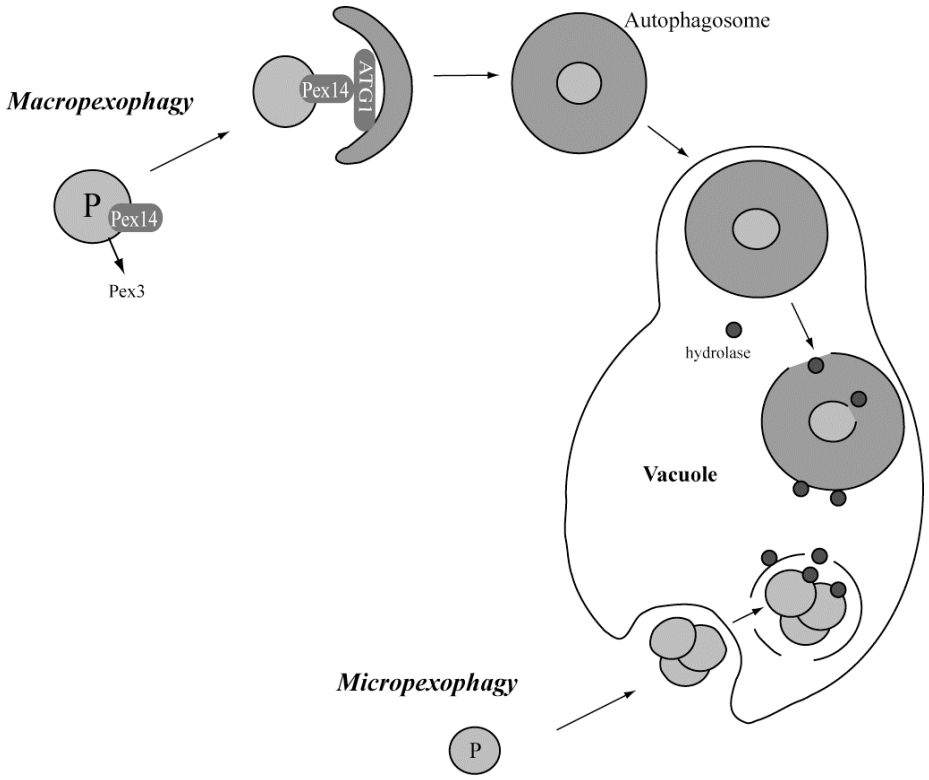


Figure 2. Degradation pathway of peroxisomes in yeast. Macropexophagy is used for the degradation of mature peroxisomes; Pex3 is removed from the membrane to allow the ATG1 machinery to dock with Pex14. After fusing with the membrane, hydrolases degrade the organelle. In micropexophagy, pre-peroxisomes are engulfed by the vacuole and subsequently degraded.

Pharmaceutical Induction of Peroxisome Proliferation

The pharmaceutical manipulation of peroxisome number has a long and interesting history. In the 1970s, research was conducted on fibrates, a class of drugs that were found to be effective in the treatment of hyperlipidemia. Early work in rodents showed that, in addition to the hypolipidemic effect observed in animals given fibrates, there was also a marked increase in the numbers of peroxisomes in their livers. Reports of hepatotoxicity and liver carcinomas in rats and mice treated chronically with fibrates raised concerns as to the safety of administration in humans (Reddy et al. 1979), however, no such effect was seen in human patients taking these compounds. Current theories of why fibrate drugs cause peroxisome proliferation in rodents but not humans include a species difference in the activation of peroxisome proliferator activated receptor (PPAR) subtypes. As the identities of endogenous ligands for the different classes of PPARs continue to be discovered, adding to the well-known fatty acids and prostaglandins (Berger et al. 2005), it is possible that a variation in ligand affinity between rodents and humans exists that leads to the observed effect. Other theories include a reduced signaling potential in humans compared to rodents, or differences in DNA binding elements (Palmer *et al.* 1998, Peters *et al.* 2005). Recent studies reported transgenic mice engineered to possess the human form of PPARα, the most widely studied subtype of the PPAR receptor (Cheung et al. 2004). These animals were resistant to the fibrate induced hepatotoxicity (Morimura et al. 2006) and lacked expression of numerous genes seen with wild-type controls, suggesting that there are differences in

genes regulated through PPARα activation between humans and mice (Gonzalez & Shah 2008).

Once activated, the PPAR receptor heterodimerizes with retinoid X receptor (RXR) and binds to DNA upstream of target genes involved in lipid metabolism, lipid transport and fatty acid β-oxidation. In regards to peroxisome proliferation, the currently available evidence supports the idea that PPAR drugs lead to an up-regulation in the fission pathway, through the actions of Pex11. Two isoforms of Pex11 have been identified, Pex11α and Pex11β. Pex11β is unresponsive to the peroxisome proliferator drug clofibrate and is believed to control constitutive peroxisome division while Pex11α regulates peroxisome abundance in response to a variety of stimuli (Schrader et al. 1998). However, examination into Pex11α knockout revealed that while PPARα does upregulate Pex11α expression, peroxisome proliferation via PPARα drugs still occurs in the Pex11α -/- model. As overcompensation via Pex11β was ruled out, the authors speculate the proliferation may be occurring through an indirect PPARα-mediated metabolic pathway (Li et al. 2002).

The Role of Peroxisomes in Cholesterol Metabolism

Cholesterol is a major component of the plasma membranes of all animal cells that controls the fluid properties of the membrane and regulates its thickness (Yeagle et al. 1990). Cholesterol also acts as a precursor for steroid hormones and is necessary for the formation of lipid rafts, which are microdomains of protein scaffolds involved in cell signaling (Gimpl & Gehrig-Burger 2007). Cholesterol levels in cells are tightly regulated,

and a fine balance is maintained between local synthesis from acetyl-CoA, uptake from the environment through cholesterol transporters in the plasma and degradation/excretion.

The regulation of cholesterol metabolism involves multiple intracellular sites. For example, dietary cholesterol is taken up via endocytosis and both endosomes and lysosomes are intimately involved in cholesterol transport and degradation (Xie *et al.* 1999, Ishibashi *et al.* 1994). The initial oxidation of cholesterol to produce hydroxycholesterol occurs on the ER membrane through the action of various cytochrome P450 enzymes (Lund *et al.* 1999, Russell 2003). Hydroxycholesterols are less lipophilic and comprise the metabolic inputs for the degradation of cholesterol to bile acids (Kessel et al. 2001). The efflux of cholesterol and its metabolites (hydroxylated cholesterol) occurs at the plasma membrane through the coordination of ATP-binding cassette transporters ABCA1 and ABCG1 with lipoproteins in the extracellular space, such as apoE; transportation to the liver occurs via high density lipoprotein (HDL) (Tall 2008). Within the liver, the cholesterol can be recycled back to cells or metabolized into bile acid and excreted through the intestine (Dietschy & Turley 2004).

The ER and mitochondria are major locations for cholesterol synthesis in cells with minor steps occurring in the cytosol. Whether peroxisomes are also involved is controversial. Kovacs and colleagues have reported that peroxisomes are important sites of the early biochemical steps in *de novo* cholesterol biosynthesis in cells. Specifically, their data indicate that the “pre-squalene” steps in cholesterol biosynthesis occur inside the peroxisome lumen. With the exception of squalene synthase, all of the enzymes necessary for cells to perform the pre-squalene steps in cholesterol synthesis have been localized within peroxisomes or found to contain conserved PTSs (Olivier et al. 2000); of

particular note is HMG-CoA reductase, the rate limiting enzyme, which is found in the ER as well as in peroxisomes (Keller et al. 1985). Peroxisomal participation in cholesterol synthesis was also demonstrated using the mouse model of ZS, the Pex2 knockout, which has severely reduced functional peroxisome numbers. In this model, there was a 40% reduction of cholesterol in liver cells, as well as a 2-fold increase in activation of the sterol regulatory element binding protein (SREBP) pathway that induces expression of enzymes in the cholesterol synthesis pathway, such as HMG-CoA reductase (Kovacs et al. 2004). It is believed that the inability of these enzymes to be imported into peroxisomes affects their catalytic efficiency.

Hogenboom and Waterham, however, have challenged the idea that peroxisomes contribute significantly to the pre-squalene steps in cholesterol biosynthesis (Wanders & Waterham 2006, Hogenboom *et al.* 2004b). These authors argue that peroxisomes are dispensable for cholesterol synthesis in cells. They have reported through subcellular fractionation studies and digitonin permeabilization experiments the inability to demonstrate peroxisomal localization of various cholesterol synthesis enzymes, such as mevalanate kinase (Hogenboom et al. 2004a). However, Kovacs et al then preformed immunofluorescent microscopy, current permeabilization techniques and isotope labeling within both Chinese hamster ovary (CHO) cells and human HepG2 cells to contradict the reports that peroxisomes are not involved. In addition to validating the peroxisomal localization of six pre-squalene enzymes, this group also established that acetyl-CoA derived from peroxisomal β-oxidation of VLCFAs remained within the peroxisome for cholesterol synthesis as opposed to being shuttled to the cytosolic acetyl-CoA portion for mitochondria assembly (Kovacs et al. 2007).

Specialized Cholesterol Metabolism in the Brain

One major difference between cholesterol in the periphery and the brain are its origins. In the periphery, dietary intake accounts for the majority of circulating cholesterol. Dietary and blood cholesterol cannot cross the blood-brain barrier, yet the brain is the most cholesterol-rich organ in the body (Bjorkhem & Meaney 2004). Thus, all brain cholesterol must be synthesized locally. Alterations in cholesterol levels, both in the brain and in the periphery, are associated with numerous disease states including AD, NPC and atherosclerosis. Cholesterol occurs in two main pools in the brain. The slowly turning over pool accounts for the majority of brain cholesterol, owing to the high concentrations required in the oligodendrocyte membrane to make myelin (Muse et al. 2001). Estimates of the lifetime of the cholesterol in the slow pool indicate a half-life of 6-12 months (Ando et al. 2003). A smaller but far more dynamic pool occurs predominantly in astrocytes, which produce nearly three times the cholesterol than neurons. Cholesterol created in astrocytes is released onto nearby neurons (Figure 4). Glia-derived cholesterol is critical for synaptogenesis (Mauch et al. 2001). To maintain cholesterol homeostasis in the brain, excess cholesterol is metabolized to 24(S)-hydroxycholesterol (24(S)-OH) which can freely reach the periphery (Dietschy & Turley 2001). CYP46, the cytochrome P450 responsible for generating 24(S)-OH, is found only in neuronal cells (Lund et al. 1999). This enzymatic degradation of cholesterol completes the metabolic coupling between neurons and astrocytes, as the 24(S)-OH can either be excreted into the cerebrospinal fluid or taken up by astrocytes in a feedback loop. Indeed, blood and CSF levels of 24(S)-OH are indicative of rates of cholesterol turnover in brain (Bretillon et al. 2000).

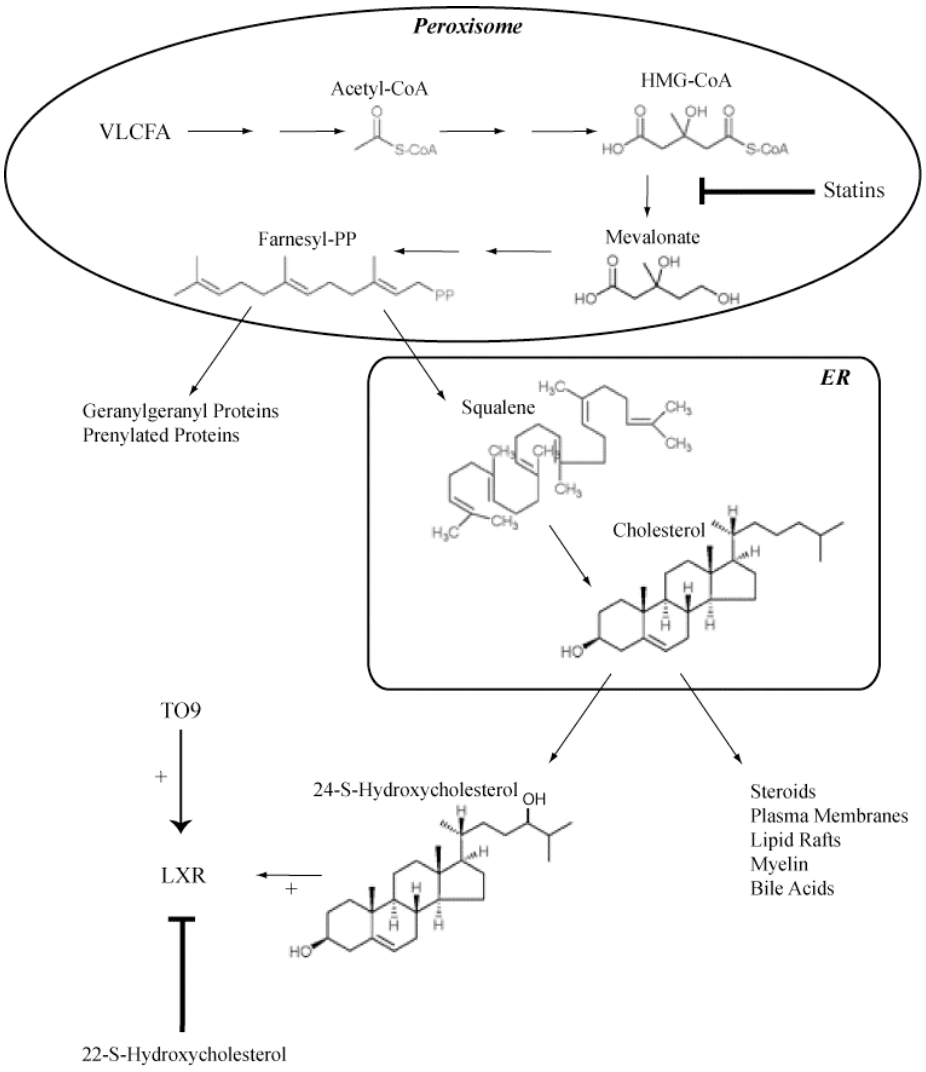


Figure 3. Putative role of peroxisomes in cholesterol metabolism. Subcellular localization shows the pre-squalene steps occurring within the peroxisome, followed by the shuttling of FPP to the ER for conversion to cholesterol. Carbon labeling of VLCFAs provided evidence that acetyl-CoA derived from β-oxidation is moved directly into cholesterol synthesis in the peroxisome.

The pre-squalene steps in peroxisomes are important not only for cholesterol synthesis, but also play a critical role in learning and memory. Farnesyl-PP, a peroxisomal pre-squalene intermediate, is converted to geranylgeranyl-PP by the enzyme farnesyl trans transferase; this product represents a nonsteroidal isoprenoid which cannot

be reintroduced to the cholesterol pathway (Wang et al. 2008). Small GTP proteins such as Rab, Rac and Rho regulate vesicular transport with membrane interactions after activation with a geranylgeranyl group (Sinensky 2000). When the synthesis of geranylgeraniol is blocked in the CYP46 knockout mouse, the resulting phenotype includes reduced spatial and motor learning in addition to deficient long-term potentiation (Kotti et al. 2006).

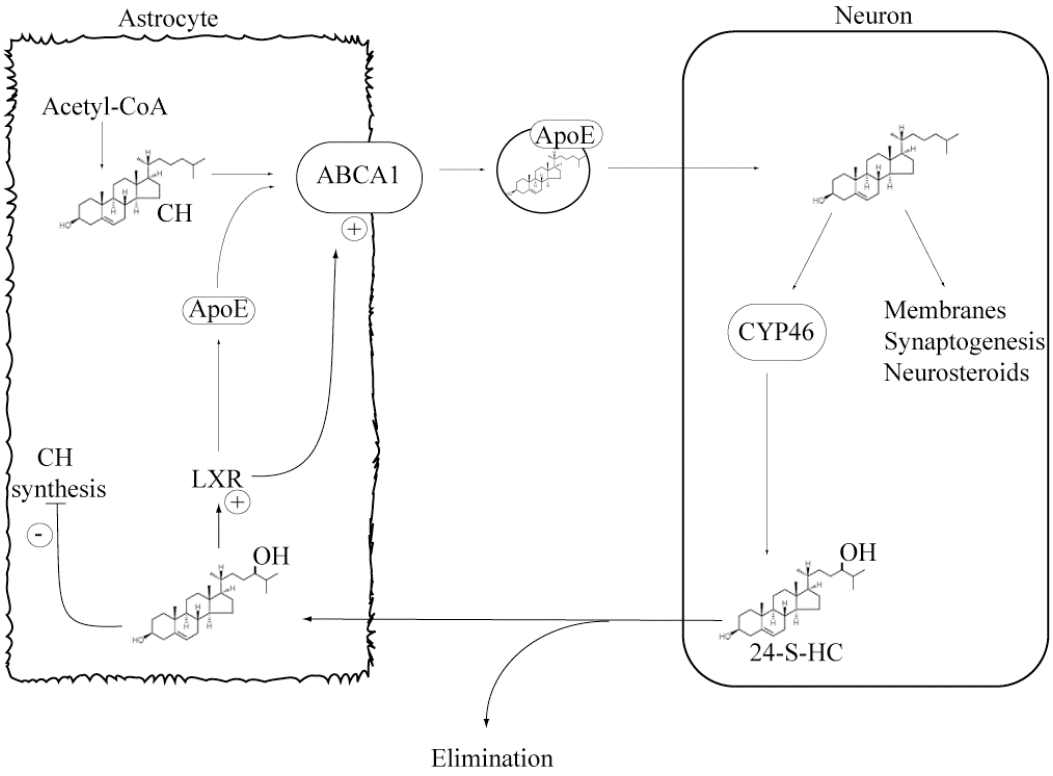


Figure 4. Specialized cholesterol handling in the brain. Acetyl-CoA derived from VLCFA β-oxidation is utilized for cholesterol synthesis within astrocytes. Efflux occurs through the plasma membrane bound ABCA1 transporter where apolipoproteinE accepts the sterol and shuttles it to the neuron. There, degradation occurs through the cytochrome P450, CYP46 resulting in 24-S-hydroxycholesterol (24(S)-HC). 24(S)-HC is then removed from the brain via the CSF or taken back up by the astrocyte where it acts as an agonist of the LXR receptor, prompting the upregulation of the ABCA1 transporter. It is also under investigation whether 24(S)-HC also acts as an inhibitor of further cholesterol synthesis via unknown mechanisms.

A Role for the Liver X Receptor

Due to the well-known deleterious affects of elevated blood cholesterol levels on cardiovascular health, the pharmaceutical reduction of cholesterol levels has been at the forefront of drug research. In addition to the use of fibrate drugs (which function at PPARα agonists), statins are among the most widely prescribed drugs in use today (Golomb 2005). Statins function by inhibiting HMG-CoA reductase and thus are very effective in treating hypercholesterolemia. Whether statins are also efficacious for treating AD is currently under investigation. Lovastatin, for example, is reported to inhibit the β-cleavage of the Aβ precursor protein (Won et al. 2008). Some statins cannot cross the BBB, while others do so only poorly; so further drug development is aimed at producing new varieties of statins with higher brain selectivity.

Similar to the PPARs, the liver X receptors (LXRs) are nuclear receptors that control lipid metabolism (Figure 5). While the PPARs mainly affect genes involved in fatty acid metabolism, the LXRs regulate genes controlling cholesterol efflux as well as other metabolic pathways (Steffensen & Gustafsson 2004). Following receptor activation, LXR and RXR form a heterodimer and bind to DNA at LXR-response elements to regulate target genes including ABCA1 (Gelissen et al. 2006). Likewise, SREBP-1c, a master cholesterol and hydroxycholesterol response protein inside cells, was found to be a target gene of LXR, the induction of which results in fatty acid synthesis (Shimano et al. 1999). It is important to note that while PPARα and LXR both form heterodimers with RXR to induce gene expression, there is no evidence that these two pathways antagonize each other through competitive usage of shared RXR receptors. In fact, both pathways

are capable of inducing genes that affect synthesis and degradation of fatty acids/sterols concurrently, although the benefit of a potential double activation is currently unknown (Anderson et al. 2004). Peroxisome proliferator response elements in the promoter regions of LXR genes are conserved across species (Tobin *et al.* 2000, Laffitte *et al.* 2001).

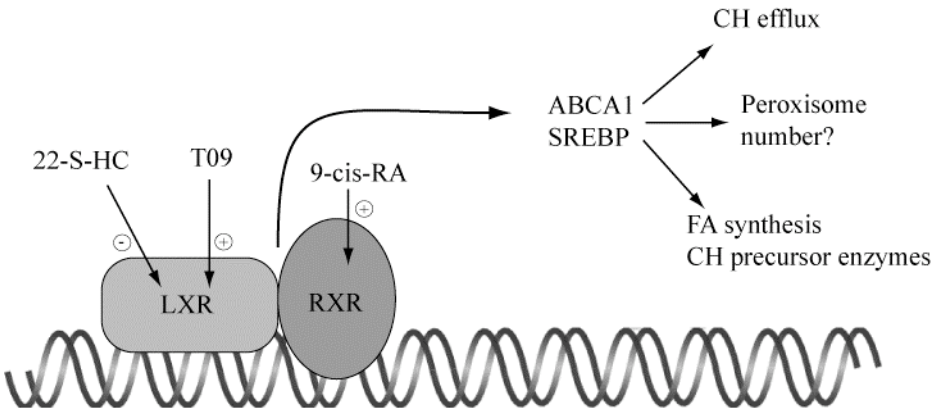


Figure 5. LXR control of cholesterol metabolism. After activation via agonists, LXR forms a heterodimer with RXR to initiate gene transcription of both SREBP and ABCA1. This results in increased cholesterol efflux, synthesis of fatty acids and the production of cholesterol precursor enzymes, such as HMG-CoA synthase and squalene synthase.

The identity of the endogenous ligands for LXRs is uncertain but endogenous oxysterols such as 24(S)-OH (Janowski et al. 1996), are the most likely candidates. Both forms of LXRs, LXRα and LXRβ, are found within the CNS (Whitney et al. 2002). Mice lacking both LXRα and β illustrate the crucial functions of these genes on the CNS (Wang et al. 2002). These animals exhibit smaller lateral ventricles, ependymal and neuronal cell loss, glial proliferation, and accumulate lipid droplets in their ependymal

cells. Motor neuron loss also occurs in these animals due to lipid accumulation. The loss resembles symptoms seen in patients with NPC (Andersson et al. 2005).

The application of endogenous ligands of LXR as therapeutic tools has been hindered somewhat by their promiscuity and poor potency. Synthetic drugs have thus been developed, which act in a more selective manner and these have shown promise for treating neurodegenerative disorders such as AD and NPC. The benefits are thought to be associated with cholesterol efflux. Treatment of astrocytes with the LXR agonist T0901317 (T09) increases the movement of cholesterol into the extracellular space through ABCA1 transcription and the expression of apolipoprotein E (apoE) in both human astrocytoma and mouse cell culture (Whitney et al. 2002). Similar to results seen with statin treatments, the application of T09 in cell culture models of AD led to decreased levels of β-amyloid (Sun et al. 2003). In the NPC model, T09 reduced the build-up of cholesterol in cells through ABCA1 expression and prolonged life (Repa et al. 2007).

Recent studies have reported that LXR agonists can induce Pex proteins and also proteins involved in fatty acid metabolism in peroxisomes (Eckert *et al.* 2007, Goodwin *et al.* 2008). Interestingly, some of these studies indicate increases in Pex16, Pex19, and Pex7, which are protein components of the de novo pathways of peroxisome biosynthesis (Steffensen et al. 2004). Thus, indirect evidence suggests that LXR agonists may influence both peroxisome proliferation and metabolism and that the proliferation of peroxisomes might be regulated by LXR.

Given the role of peroxisomes in lipid metabolism and their clearly established relevance to disease states, we decided to evaluate astrocyte cultures treated with a

variety of drugs to discover whether peroxisomes were affected and if they played a part in any resulting changes within the cell. To this end, we developed a novel morphological assay for quantifying peroxisomes in cells. Our initial attempts to induce peroxisome proliferation via the canonical PPARα pathways were unsuccessful, but we serendipitously discovered that LXR agonists functioned as a novel class of peroxisome proliferators in astrocytes. Since LXR-targeted therapies are in development for treating brain diseases—we decided to pursue the novel LXR-mediated pathways of peroxisome proliferation in an attempt to discern the molecular mechanism and relationship to cholesterol metabolism in brain.

Liver X receptor induces peroxisome proliferation in astrocytes

Megan R. Hershfield, Yoanna S. Pumplalova and Michael J. Schell

Department of Pharmacology, Uniformed Services University, Bethesda, MD 20814 USA

Address correspondence and reprint requests to Michael J. Schell, Ph.D. E-mail mschell@usuhs.mil

Abbreviations used: 4-PBA, 4-phenylbutyric acid; Acox, acyl-CoA oxidase; ApoA1, apolipoprotein A1; AD, Alzheimer's disease; PPARα, peroxisome proliferator activated receptor alpha; T09, T0901317; 22(S)-HC, 22(S)-hydroxycholesterol.

Abstract

Peroxisomes are organelles that are the sites of specialized metabolism in all eukaryotic cells. The pharmaceutical regulation of peroxisome abundance in cells has potential for treating some brain diseases, but the mechanisms that control peroxisome number in brain cells are largely unknown. Furthermore, the relationship between peroxisomes and the disposition of cholesterol is unclear. Using primary cultures of rat cerebral cortical astrocytes we have identified a novel means of regulating the abundance of peroxisomes via the liver X receptor (LXR), a nuclear receptor that controls cholesterol efflux in cells. Treatment of cultures with T0901317, an agonist at the LXR, increased the peroxisomal content of astrocytes by 20%, while treatment with 22(S)-hydroxycholesterol, an antagonist, reduced peroxisomes by 33%. Western blots indicated that T0901317 increased levels of the LXR-regulated protein ABCA1 and enhanced cholesterol efflux. The effect of LXR ligands was independent of peroxisome proliferator receptor alpha (PPARα), as PPARα gene products were not induced. Furthermore, PPARα agonist treatment did not affect peroxisome abundance in astrocytes. Lovastatin, an inhibitor of cholesterol biosynthesis, also induced peroxisome proliferation in cells. Our data suggest a novel relationship between cellular cholesterol homeostasis and peroxisome number in brain cells and reveal the potential for selective mechanisms to control organelle numbers in brain cells.

Keywords: cholesterol, nuclear receptor, hydroxysterol, fibrate, organelle, statin

Running title: peroxisome proliferation in astrocytes

Peroxisomes are the sites of specialized oxidative metabolism in cells, including the oxidation of very long chain fatty acids, biosynthesis of plasmalogen ether lipids, the production of bile acids, and the degradation of D-amino acids (Schrader & Yoon 2007). Genetic deficiencies in peroxisomal metabolism lead to serious disorders in humans, which manifest prominently in brain cells (Gould & Valle 2000). Despite this, the contribution of peroxisomes to cellular metabolism in brain cells is poorly understood. In situations where the specialized metabolic pathways of peroxisomes are reduced or absent, it may be advantageous to induce peroxisomal metabolism or even increase the number of organelles themselves (Wei *et al.* 2000).

Several pharmaceutical strategies for increasing peroxisome numbers in brain cells have been suggested as potential therapies for disorders of peroxisome biogenesis and function; however the efficacy of such therapies in human patients is unproven (Wei *et al.* 2000, Reddy & Chu 1996). Classically, the proliferation of peroxisomes has been induced by fibrate drugs via mechanisms acting through the peroxisome proliferator activated receptor alpha (PPARα), a nuclear receptor. However, peroxisome proliferation through this pathway may be species-specific and does not occur in humans (Gonzalez & Shah 2008). Pharmaceutically induced peroxisome proliferation occurs in human cells following exposure to 4-phenylbutyrate through a mechanism that may involve histone deacetylase regulation, but the high doses required and extensive drug metabolism limit the effectiveness of such therapies in patients (Gondcaille *et al.* 2005).

Cholesterol metabolism in brain is independent of blood cholesterol and cholesterol homeostasis in the periphery. Brain cells synthesize all of their cholesterol *de novo* and the complex cellular exchange of cholesterol, its precursors, and its metabolites has been linked to synaptogenesis (Mauch *et al.* 2001) learning and memory (Kotti *et al.* 2006, Ramirez *et al.* 2008) and the pathological processes leading to Alzheimer's disease (AD) (Bjorkhem & Meaney 2004) and Neiman-Pick type C disease (Repa *et al.* 2007). In addition to the established roles for peroxisomes described above, these organelles have been suggested to be crucial participants in the early steps of cholesterol biosynthesis, but this remains controversial. Compelling arguments have been made both for (Kovacs *et al.* 2007, Kovacs *et al.* 2004) and against (Wanders & Waterham 2006, Hogenboom *et al.* 2003) the hypothesis that peroxisomes are specialized sites of the pre-squalene steps in the *de novo* pathway of cholesterol biosynthesis. Interestingly, humans afflicted with X-linked adrenoleukodystrophy (X-ALD), a disorder of peroxisome-specific metabolism, show disturbances in cholesterol metabolism (Weinhofer *et al.* 2005).

The liver X receptor (LXR) is a nuclear receptor that may provide novel therapeutic approaches to AD and other neurodegenerative disorders because it has the potential to be used as a cholesterol-regulating drug target in brain cells. LXR exerts its effects on cholesterol homeostasis largely via its ability to control the rate of cholesterol efflux from cells. Regulation of cholesterol efflux through LXR-controlled mechanisms affects the processing of amyloid precursor protein (Burns *et al.* 2006). Furthermore, a recent study in which rats were injected with an LXR agonist showed that levels of HMG CoA reductase, the rate-limiting step in cholesterol biosynthesis, were increased in a peroxisome-enriched fraction isolated from brain tissue (Eckert *et al.* 2007). Thirdly,

LXR agonists are known to increase peroxisome-exclusive pathways of long-chain fatty acid oxidation via increasing the transcription of the relevant enzymes (Hu *et al.* 2005), providing direct evidence for the LXR-regulated control of peroxisomal metabolism. These and other studies point to a metabolic relationship between cholesterol biosynthesis and peroxisomes in brain.

In this study, we identified a previously unknown means of controlling peroxisome number in astrocytes via drugs that affect cholesterol homeostasis. We show that peroxisome abundance is affected by LXR-targeted molecules and also by lovastatin, an inhibitor of HMG CoA reductase. The selective nature of LXR-induced peroxisome proliferation suggests that LXR-targeted drugs may simultaneously regulate both organelle number and cholesterol biosynthesis pathways in brain cells.

Materials and methods

Animals

All procedures on postnatal Sprague-Dawley rat pups (Taconic, Rockville, MD) were performed in accordance with the guidelines of the USUHS Institutional Animal Care and Use Committee.

Antibodies

Anti-PMP70 (Sigma, P0497, directed against a synthetic peptide corresponding to amino acid residues 644-659 of rat PMP70): 1:200 for immunofluorescence, 1:1000 for WB; anti-ABCA1 (Chemicon, Clone AB.H10) 1:1000; anti-ABCD2 (Santa Cruz, 25353) 1:500; anti-Acox (Abnova, H00000051-B01) 1:1000; anti-DLP1 (Abnova, Clone: 3B5), 1:1000; anti-βactin (Abcam, clone: 8226) 1:20000; anti-ATP synthase (Chemicon, Clone: 4.3E8.D1) 1:100; anti-endolyn (clone 501; a gift from Dr. Gudrun Ihrke) 1:100.

Cell cultures and drug stimulation

Glial cultures were prepared from neonatal rats (0-48 hr after birth) using a modification of a previously described protocol (Levison & McCarthy 1991). Briefly, cerebral cortices were removed, meninges excised and cells dissociated in trypsin for 20 min. Tissue was triturated through needles of decreasing gauge, filtered through a nylon mesh, and plated into poly-D-lysine coated tissue culture flasks. Cells were grown in DMEM medium (Invitrogen Cat #11965) containing 10% fetal bovine serum and supplemented with penicillin-streptomycin, extra serine and pyruvate. Flasks and plates were incubated

at 37°C in 5% CO_2, and media was changed every 3 days. At day 10, cultures consisted of approximately 75% glial fibrillary acidic protein (GFAP)-positive cells with a flat, fibroblastic morphology. Flasks of cells at day 7-10 in vitro were trypsinized and plated onto poly-D-lysine coverslips. Two to 3 days after re-plating, cells were treated with T0901317 (T09; 250 nM; Cayman), lovastatin (50 nM; Cayman), 4-phenylbutyrate (5 mM, Sigma), and/or 10 μM 22(S)-hydroxycholesterol (22(S)-HC; Sigma). The vehicle for T09, lovastatin, and 22(S)-HC, WY14643 (10 μM; Tocris) and GW7647 (6 nM; Tocris) was 0.1% ethanol; vehicle for 4-PBA was PBS. FAO cells (gift from Dr. Peter Kim) were grown in F12 Ham's medium (Kaighn's modification; Sigma, N3520) with 10% fetal bovine serum, and penicillin-streptomycin. The drug treatment protocol was identical to that used for glial cultures.

Immunocytochemistry

Cells grown on coverslips were washed twice in PBS plus divalent cations and then fixed in 4% paraformaldehyde/0.1 M NaPPi, pH 7.4 for 20 min at room temperature. All further steps were carried out in PBS without divalent cations. Following 5 washes, cells were permeabilized in 0.1% Triton X-100 for 8 min and blocked in 2% fish skin gelatin (Sigma). Primary antibodies were diluted in 1% fish skin gelatin and incubated with the coverslips at 4°C overnight. After washing, cells were re-blocked in PBS containing 2% fish gelatin for 15 min prior to incubation with Alexa fluor-tagged anti-rabbit or anti-mouse IgG (1:200; Invitrogen) for 30 min at room temperature. Cells were mounted in Prolong Gold antifade with DAPI (Invitrogen) and air-dried at least 24 hr before imaging.

Fluorescent imaging of cellular cholesterol was achieved with a filipin-based cholesterol detection kit (Cayman), according to the manufacturer's instructions.

Imaging, organelle counting, and statistical analysis

In preliminary studies, we established that cells in our cultures exhibiting a fibroblastic morphology were always positive for the astrocyte marker glial fibrillary acidic protein (GFAP), and thus were likely to be type 1 astrocytes. For this study, we restricted our analysis to this type of cell. Peroxisomes were labeled with an antibody against PMP70, a universal resident membrane protein of peroxisomes. In preliminary double-staining experiments we established that PMP70-positive structures in cells were in 100% correspondence with a second peroxisomal marker (acyl-CoA oxidase, Acox). An investigator blind to the treatment condition performed image collection and assays of peroxisome abundance. Images were collected using a 63X (N.A. 1.4) objective on a Zeiss Axiovert 200 widefield microscope equipped with a CCD camera and a piezo z-stage (ASI). Acquisition was controlled via Volocity (Improvision), which set camera exposures automatically. For each astrocyte analyzed, 8 slices over a total z-distance of 3 μm through the cell were captured and then deconvolved using the Volocity rapid restoration algorithm. Image stacks were then exported to ImageJ (Wayne Rasband, NIH) and flattened to maximal projections. Square areas of 100 μm^2 were auto-thresholded and then quantified using ImageJ's particle analysis routine, which measured both peroxisome size and number. For each drug condition in each experiment, 27 regions of interest from 9 different astrocytes were analyzed and the results from 3 or more separate experiments were pooled. Graphpad Prism was used for statistical testing

to compare groups via one-way ANOVA with Dunnett's post hoc analysis. In preliminary experiments we found no difference in peroxisome numbers between untreated controls and vehicle controls containing 0.1% ethanol. For analysis of peroxisomes in FAO cells, which are thicker, we collected 32 slices per cell over a z-distance of 8 μm before analysis of 625 μm^2 areas, which were then analyzed in the same manner as the astrocytes.

Hi-resolution imaging of peroxisome morphologies

Images of PMP70-labeled peroxisomes were collected on a the Zeiss Axiovert widefield microscope equipped with a 100x objective (N.A. 1.4). Ten Z-sections, spaced over a total Z-distance of 3 μm. were subjected to the rapid restoration deconvolution routine in the Volocity software package. Deconvolved stacks were then exported to ImageJ and flattened as maximal projections. Representative square areas of 3.42 um^2 were collected as montages to illustrate peroxisome morphology. These images were optimally contrasted before collation in Adobe Photoshop.

Western blot analysis

Following drug treatments as described above, cells were solubilized in RIPA buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS, phenylmethylsulfonyl fluoride (PMSF), and a phosphatase inhibitor cocktail (Calbiochem). Equal amounts of protein (10 μg) were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Membranes were blocked in non-fat milk with 0.05% Tween-20 for one hour, followed

by incubation with primary antibody in milk overnight at 4°C. After washing, membranes were further incubated with a horseradish peroxidase (HRP) secondary antibody (Pierce) for one hour at room temperature. Membranes were then washed, treated with ECL femto substrate (Pierce) and chemiluminescence was captured with Fuji camera. Band intensities were quantified using gel band analyzer in ImageJ and statistically tested as described above.

Results

PPAR agonists do not induce peroxisome proliferation in rat astrocytes.

To measure peroxisome abundance in cells, we developed a morphological assay for peroxisomes labeled with the marker protein PMP70. The best-characterized system of peroxisome proliferation involves the induction of peroxisomes in rodent hepatocytes with fibrates, which are thought to act as agonists at PPARα. A rat hepatocyte cell line FAO was treated with the PPARα agonists GW7647 and WY14643 for two days and then cells were fixed and analyzed for peroxisome abundance and size (Fig. 1). Consistent with previous studies, we observed clear changes in peroxisome number and morphology in hepatocyte-derived cells following treatment with PPARα agonists (Fig. 1 a-f). The effect of the two PPARα agonists on peroxisomes was not identical. Cells treated with GW7647 exhibited increased size (Fig. 1c and e) in the fraction of cellular area occupied by peroxisomes (Fig 1f). By contrast, WY14643 treatment caused an increase in peroxisome size, but an apparent decrease in total number of peroxisomes per

cells. Both drugs caused an obvious change in peroxisome morphology compared to controls (Fig. 1a versus 1b and 1c). In cells treated with WY14643 especially, peroxisomes became more tubular and exhibited a large number of toroid shapes, with a distinctive "keyhole" morphology (Fig. 1b). This change was also observed to a lesser extent in the GW7647-treated cells, and was never observed in controls. These data show that hepatocyte cells respond to PPARα agonists as previously described (Lee *et al.* 1995), thus validating our morphological assay for peroxisomes.

However, when analogous experiments were performed on primary cultures of rat glia, we did not observe a significant change in peroxisome abundance, size or morphology (Fig. 1 g-l). Peroxisomes in control astrocytes were slightly smaller on average than those in control FAO cells, and they never exhibited the distinct toroid shapes seen in the FAO cells after treatments with PPARα agonists (Fig 1g versus 1h and 1i). Low levels of PPARα in astrocytes might explain our inability to induce peroxisome proliferation in these cells. Although PPARα has been reported to occur in brain (Michalik *et al.* 2002, Cullingford *et al.* 1998), examination of the Allen Brain Atlas revealed that PPARα expression is relatively low in brain (Lein *et al.* 2007). We therefore explored alternative transcriptional signaling systems that might operate in brain to control peroxisome abundance.

LXR drugs influence peroxisome number in astrocytes

Fibrates are prescribed to improve blood lipid profiles by lowering triglycerides and increasing synthesis of certain apolipoproteins (Vosper *et al.* 2002). Another class of drugs capable of modulating blood lipids are those acting at the LXR, which controls

cholesterol efflux from cells (Zhang & Mangelsdorf 2002). We wondered if LXR drugs might also affect peroxisome abundance (Fig. 2). The affinity of the agonist T09 for LXR is approximately 20 nM (Mitro *et al.* 2007). For these studies we utilized the antagonist 22(S)-HC, the unnatural isomer of 22-hydroxycholesterol, as previously reported (Kase *et al.* 2007). We thus treated rat astrocyte cultures with 250 nM T09, 10 μM 22(S)-HC, or both for various times prior to morphological analysis of peroxisomes (Fig. 2a). The cellular area occupied by peroxisomes peaked after 48 hr of treatment with T09, with no further increase observed at 72 hr. By contrast, 22(S)-HC caused a diminution in peroxisomes with a similar time course. We adopted a 48 hr drug treatment regimen for all further studies.

When cultures were treated with the LXR agonist T09 (250 nM, 48 hours), we observed a significant increase both in peroxisome size and number (Fig. 2 f-h). The calculated peroxisome area following T09 treatment for two days reached 136% of controls. By contrast, the LXR antagonist 22(S)-HC, reduced peroxisome size and number compared to controls (Fig. 2 f-h). Furthermore, the peroxisome proliferative effects of T09 could be prevented by the inclusion of 22(S)-HC. We carefully examined the organelle morphology under these conditions and saw no differences across groups, unlike the changes seen in FAO cells following exposure to PPARα-targeted drugs. These data suggest that the increase in peroxisome abundance occurs via an LXR-regulated transcriptional mechanism, which has not been described previously.

The density of mitochondria is not influenced by LXR.

We wondered if the proliferation of peroxisomes observed was organelle-specific, therefore we measured the abundance of mitochondria under conditions that caused maximal and minimal peroxisome proliferation in astrocytes. Astrocytes were labeled with an antibody to the mitochondrial marker ATP synthase, using a different fluorescence channel on the same coverslips used for the peroxisome measurements. Neither T09 nor 22(S)-HC caused a significant change in mitochondrial area (Fig. 3). In other experiments, we tested whether 22(S)-HC was stimulating pexophagy, the degradation of peroxisomes in lysosomes. After a 2-day incubation in T09 or 22(S)-HC, we co-labeled cells with antibodies to PMP70 and endolyn, a marker of late endosomes and lysosomes (data not shown). Three-dimensional deconvolution microscopy revealed no detectable overlap between the two markers under any conditions. Thus, the effects of LXR ligands showed selectivity for peroxisomes over other organelles.

Lovastatin also influences peroxisome number

Since LXRs are known to influence the handling of cellular cholesterol and because some studies have implicated the peroxisome as an important cellular location for the early steps in cholesterol biosynthesis (Kovacs et al. 2004), we tested whether other drugs that affect cellular cholesterol might also influence peroxisome number. Statins influence the handling of cholesterol by inhibiting the enzyme HMG CoA reductase, which is the rate-limiting step in the *de novo* synthesis of cholesterol from acetyl CoA. The Km of lovastatin for its target is about 20 nM (Goldstein & Brown 1990). When astrocytes were treated with 50 nM lovastatin for various times, peroxisomal area increased steadily, reaching about 140% of controls by 72 hr (Fig. 4a). Inclusion of 22(S)-HC during the

lovastatin time course reversed the peroxisome proliferation and caused a diminution in peroxisome number (Fig. 4 a). We adopted the 48 hr lovastatin exposure for further experiments in order to better compare our results with those obtained with T09. Lovastatin affected peroxisomal number in manner similar to TO9 in that we observed a significant increase in peroxisome number and total area, but no change in peroxisome size (Fig. 4 e-g). Surprisingly, the peroxisome proliferative effects of lovastatin were reversed in the presence of 22(S)-HC, the LXR antagonist (Fig. 4 g). As with T09 and 22(S)-HC, organelle morphology did not change appreciably.

22(S)-HC and statins influence peroxisome number in FAO hepatocytes

We wondered if the ability of LXR to regulate peroxisome abundance was generalizable to other cell types. We thus tested the hepatocyte FAO cell line for LXR-induced changes in peroxisomes (Fig. 5). In contrast to astrocytes, FAO cells showed no significant increase in peroxisome abundance when treated with 250 nM T09. However, 22(S)-HC did significantly reduce peroxisome number and area without affecting the average size of peroxisomes in these cells. We also tested the effect of lovastatin on peroxisomes in FAO cells and found a modest but significant increase over controls. These data suggest that effects of T09 on peroxisome abundance are cell-type specific, perhaps owing to differences in the cellular production of hydroxysterols or other endogenous LXR agonists. By contrast, 22(S)-HC and lovastatin appear to be more generalized regulators of peroxisome abundance. These studies reinforce the evidence that the disposition of cholesterol in cells can influence peroxisome abundance.

LXR agonist T09 induces ABCA1 protein and activity

LXR agonists produce clinically relevant increases in cholesterol efflux via increased expression of ABCA1, the major plasma membrane cholesterol efflux pump in most cells (Whitney *et al.* 2002, Eckert et al. 2007). To confirm increased ABCA1 expression under the conditions of our study, we treated astrocytes with T09 and then analyzed ABCA1 levels by Western blot (Fig. 6a). By 48 hrs protein levels of ABCA1 had increased at least 5-fold over controls. 22(S)-HC had no significant effect on ABCA1. We also did not observe an increase in ABCA1 when cells were treated with lovastatin, an observation at odds with our earlier data that suggested lovastatin was causing the production of an endogenous LXR agonist.

We then examined the disposition of cholesterol itself, using an improved morphological assay for cholesterol based on the ability of the antibiotic filipin to reveal cellular cholesterol (Soccio & Breslow 2004). Cells treated with T09 showed increased plasma membrane staining for cholesterol (Fig. 6 d versus b), and this staining was diminished if excess apolipoprotein A1 (ApoA1; 7.5 μM, Sigma), the cholesterol efflux acceptor, was included in the extracellular medium, (Fig. 6 e). In other experiments (not shown), we found that including ApoA1 in the cell culture medium during LXR drug treatments had no significant effects on peroxisome abundance. These experiments show that T09, as expected, was a robust activator of cholesterol efflux in astrocytes (Eckert et al. 2007).

T09 does not induce PMP70 or acyl-CoA oxidase

In an attempt to define the underlying mechanism responsible for LXR-induced peroxisome proliferation, we treated astrocytes with LXR-targeted drugs and then analyzed protein levels of some candidate mediators that might explain our results. One possibility was that we were simply inducing levels of PMP70 selectively, rather than peroxisomes in general. However, levels of PMP70 did not change significantly (Fig. 7a and c). Another possibility is that LXR agonists indirectly lead to the activation of PPARα and increase transcription via PPARα-regulated gene products that then trigger an increase in peroxisome abundance. To test this, we probed with an antibody to ABCD2, a *bona fide* PPARα regulated gene product. Neither LXR targeted drugs nor lovastatin affected ABCD2 levels. By contrast, treating astrocytes with 4-phenylbutyrate, a known inducer of ABCD2, caused a greater than 3-fold increase ABCD2 levels (Fig. 7a and b). At third possible mechanism for inducing peroxisome proliferation involves increased levels of Acox protein within the lumen of the peroxisome (Guo *et al.* 2007). However, we found no evidence that our drug treatments affected levels of Acox (Fig. 7a and d). Finally, we tested levels of DLP1, a component of the machinery involved in peroxisome fission (Kobayashi *et al.* 2007). Again, no change in DLP1 levels were found (Fig. 7a and e), suggesting that that peroxisome fission, the most common mechanism for increasing peroxisome abundance, might not be activated by LXR pathways. The two most likely candidate proteins for controlling peroxisome abundance are Pex11 and Pex16, which participate in the fission or *de novo* pathways of peroxisome biogenesis respectively. Unfortunately, antibodies directed against these proteins are not currently available and thus we were unable to test them in our model. Nevertheless, our data point towards a novel means of pharmaceutically induced peroxisome proliferation

in astrocytes, which seems to occur via previously unappreciated mechanisms.

Discussion

The major finding of this study is that astrocytes, when exposed to agonists of LXR or when exposed to lovastatin, increase the abundance of their peroxisomes. Our studies reveal a novel transcriptionally based mechanism for controlling peroxisome numbers in these cells. The LXR-induced proliferation of peroxisomes was selective for astrocytes, in that it did not appear to occur in a hepatocyte cell line. Thus, our study raises the possibility of developing pharmaceutical approaches to peroxisome proliferation that are selective for glial cells. By contrast, lovastatin was effective at inducing peroxisome proliferation of FAO cells, suggesting a more universal effect on peroxisomes by statins.

What could explain the selectivity of LXR-mediated peroxisome proliferation in astrocytes? Our data does not distinguish whether LXR-induced peroxisome proliferation was a direct transcriptional effect of LXR leading to increased levels of protein machinery that controls abundance or rather was an indirect effect following the increased cholesterol efflux caused by the large enhancement in ABCA1 transcription. As application of ApoA1 to the culture media that led to an efflux of cholesterol seen with filipin staining did not enhance our peroxisome proliferative effect, further studies will be required to address this question. Hepatocytes express predominantly the LXRα isoform, but brain expresses mainly LXRβ (Zhang & Mangelsdorf 2002). While the DNA binding regions of the two LXR isoforms are almost identical, no evidence suggests

that they regulate different sets of genes. However, the regulatory pathways leading to the activation of LXRα and β are, in some cases, heterogeneous, perhaps owing to differences in ligand preference (Tobin *et al.* 2000, Chawla *et al.* 2001). Since our experiments showed that 22(S)-HC, a non-natural hydroxysterol with LXR antagonist activity, caused a diminution of peroxisomes in FAO cells, perhaps hepatocytes produce a different repertoire of endogenous LXR agonists. For example 24(S), 25-epoxycholesterol, a potent LXR agonist that is a product of one branch from the squalene portion of cholesterol biosynthesis, is very abundant in liver (Peet *et al.* 1998) but not brain (Wong *et al.* 2007). By contrast, 24(S)-hydroxycholesterol is made exclusively brain (Lund *et al.* 2003).

Our experiments showed that drugs affecting LXR are more effective triggers of peroxisome proliferation in astrocytes than are drugs targeted to PPARα. The most well studied PPARα ligands are the fibrates, which act on PPARα to induce the peroxin Pex11, a component of the peroxisome fission pathway (Schrader *et al.* 1998, Li & Gould 2002, Kobayashi et al. 2007). The PPARα effect is selective for rodents, owing to differences in DNA binding regions between species (Gonzalez & Shah 2008). Peroxisome proliferation has, however, been induced in humans with 4-PBA, which is also thought to modulate Pex11 transcription via changes in histone acetylation (Wei et al. 2000). The low levels of PPARα in brain may explain why drugs that target this nuclear receptor had no effect on peroxisomes in astrocytes. Alternatively, the molecular mechanisms that lead to changes in peroxisome abundance in astrocytes may be different. LXR regulates a host of gene products (Steffensen & Gustafsson 2004) and the full extent of its effects on lipid metabolism therapeutic potentials has not yet been explored. LXR-

based therapies have been explored for treating AD (Sun *et al.* 2003) and Parkinson's disease (Kim *et al.* 2008). Our results raise the possibility that they might also proliferate peroxisomes. Whether this property accounts for any of the beneficial effects (Abildayeva *et al.* 2006) or undesirable side effects (Schultz *et al.* 2000) deserves further investigation.

Our study also showed that lovastatin, a drug used clinically to regulate blood cholesterol, is also a peroxisome proliferator. In contrast to LXR-mediated peroxisome proliferation, we observed that lovastatin showed this effect in astrocytes and a hepatocyte-like cell line. The molecular mechanism by which lovastatin influences peroxisome abundance is not immediately obvious. We observed that lovastatin-induced peroxisome proliferation in astrocytes could be reversed by the presence the 22(S)-HC, an LXR antagonist. However, ABCA1, the main gene up-regulated by LXR agonists was not affected by lovastatin, which argues against a model whereby lovastatin produces increased levels of an endogenous LXR agonist. Our data suggest complex cross talk between LXR and pathways of cholesterol biosynthesis, perhaps related to other sterol-regulated transcriptional systems, such as the sterol regulatory binding proteins (Repa *et al.* 2000, Goodwin *et al.* 2008). A recent study suggested a direct crosstalk between LXR and enzymes controlling cholesterol biosynthesis (Wang *et al.* 2008). Lovastatin has shown benefit in the treatment of neurodegenerative disorders. Our study raises the possibility that some of its benefits (Jick *et al.* 2000) or side effects (Golomb 2005) occur via biochemical pathways involving peroxisomes. Interestingly, statins have also shown some tentative promise in treating peroxisome biogenesis disorders (Engelen *et al.* 2008, Singh *et al.* 1998).

Peroxisomes are crucial participants in the pathways of cholesterol catabolism that lead to bile acids and a need for the increased production of bile acids is one possible corollary with our discovery of a link between peroxisome abundance and cholesterol homeostasis (Ferdinandusse & Houten 2006). The importance of peroxisomes to cholesterol biosynthesis is more controversial (Wanders & Waterham 2006). Most of the enzymes in the pre-squalene steps of cholesterol biosynthesis contain putative peroxisomal targeting sequences and have been localized to peroxisomes (Kovacs et al. 2007, Aboushadi *et al.* 2000). In some (Kovacs et al. 2004), but not all (Hogenboom et al. 2003) studies, abnormalities in cholesterol biosynthesis have been reported in conjunction with mutations in peroxins. The pre-squalene biosynthetic pathways are also crucial for the production of isoprenoids, which have important signaling roles, independent of cholesterol per se. Most notably, the production of isoprene appears to regulate learning and memory processes in brain cells (Sinensky 2000, Kotti et al. 2006). Furthermore, the beneficial effects of statins in preventing AD may involve decreased Aβ generation via a reduced geranylgeranyl-pyrophosphate (GGPP) dependent endocytosis (Won *et al.* 2008). Our data indicate that two different drug classes with known effects on cholesterol homeostasis in brain also impact the number of peroxisomes in astrocytes. We have not determined whether the biosynthesis of cholesterol and/or isoprenoids is/are directly affected by changes in peroxisome numbers, but at least one study has suggested that increased peroxisome abundance may be beneficial for treating AD (Santos *et al.* 2005).

A previous study showed that LXR agonists caused the up-regulation of enzymes involved in the peroxisome-specific metabolism of long-chain fatty acids, providing direct evidence of a relationship between the targets of LXR and peroxisome-specific

metabolism (Hu et al. 2005). The present study indicates that the abundance of the organelles themselves may be regulated via LXR. What molecular mechanism could account for a transcriptionally regulated increase in peroxisomes? In addition to the Pex11-dependent pathways of peroxisome fission described above, recent studies have described a novel pathway of peroxisomal biogenesis via budding from the endoplasmic reticulum that depends on the peroxin Pex16 (Kim *et al.* 2006, Titorenko & Mullen 2006, South & Gould 1999). The relative contribution of the *de novo* pathway to peroxisome abundance is not known and the degree to which the two biosynthetic pathways for peroxisomes are used may vary among cells and tissues. Indeed, brain levels of the fission pathway mediator Pex11 are relatively low; by contrast, Pex16 levels are very high in all brain areas (Lein et al. 2007). Furthermore, microarray studies in a number of tissues indicate a clear increase in Pex16 transcription following LXR agonists (Steffensen *et al.* 2004) and mice engineered to over-express ABCA1 also exhibit enhanced Pex16 transcription (Joyce *et al.* 2006). Future studies will test the hypothesis that LXR-induced increases in peroxisomal abundance occur via the Pex16-dependent *de novo* pathway.

Acknowledgements

We thank Dr. Peter Kim for providing FAO cells and helpful discussion, Dr. Gudrun Ihrke for antibodies against Endolyn and helpful discussions. Dr. Jeremy Hershfield for assistance with data analysis. A Research Starter Award from the PhRMA Foundation to MJS, and a Summer Undergraduate Research Fellowship from ASPET and the Henry Jackson Foundation to YSP supported this work.

References

Abildayeva, K., Jansen, P. J., Hirsch-Reinshagen, V. et al. (2006) 24(S)-hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux. *The Journal of biological chemistry,* **281,** 12799-12808.

Aboushadi, N., Shackelford, J. E., Jessani, N., Gentile, A. and Krisans, S. K. (2000) Characterization of peroxisomal 3-hydroxy-3-methylglutaryl coenzyme A reductase in UT2 cells: sterol biosynthesis, phosphorylation, degradation, and statin inhibition. *Biochemistry,* **39,** 237-247.

Bjorkhem, I. and Meaney, S. (2004) Brain cholesterol: long secret life behind a barrier. *Arteriosclerosis, thrombosis, and vascular biology,* **24,** 806-815.

Burns, M. P., Vardanian, L., Pajoohesh-Ganji, A., Wang, L., Cooper, M., Harris, D. C., Duff, K. and Rebeck, G. W. (2006) The effects of ABCA1 on cholesterol efflux and Abeta levels in vitro and in vivo. *Journal of neurochemistry,* **98,** 792-800.

Chawla, A., Boisvert, W. A., Lee, C. H. et al. (2001) A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Molecular cell,* **7,** 161-171.

Cullingford, T. E., Bhakoo, K., Peuchen, S., Dolphin, C. T., Patel, R. and Clark, J. B. (1998) Distribution of mRNAs encoding the peroxisome proliferator-activated receptor alpha, beta, and gamma and the retinoid X receptor alpha, beta, and gamma in rat central nervous system. *Journal of neurochemistry,* **70,** 1366-1375.

Eckert, G. P., Vardanian, L., Rebeck, G. W. and Burns, M. P. (2007) Regulation of central nervous system cholesterol homeostasis by the liver X receptor agonist TO-901317. *Neuroscience letters,* **423,** 47-52.

Engelen, M., Ofman, R., Mooijer, P. A., Poll-The, B. T., Wanders, R. J. and Kemp, S. (2008) Cholesterol-deprivation increases mono-unsaturated very long-chain fatty acids in skin fibroblasts from patients with X-linked adrenoleukodystrophy. *Biochimica et biophysica acta,* **1781,** 105-111.

Ferdinandusse, S. and Houten, S. M. (2006) Peroxisomes and bile acid biosynthesis. *Biochimica et biophysica acta,* **1763,** 1427-1440.

Goldstein, J. L. and Brown, M. S. (1990) Regulation of the mevalonate pathway. *Nature,* **343,** 425-430.

Golomb, B. A. (2005) Implications of statin adverse effects in the elderly. *Expert Opin Drug Saf,* **4,** 389-397.

Gondcaille, C., Depreter, M., Fourcade, S. et al. (2005) Phenylbutyrate up-regulates the adrenoleukodystrophy-related gene as a nonclassical peroxisome proliferator. *The Journal of cell biology,* **169,** 93-104.

Gonzalez, F. J. and Shah, Y. M. (2008) PPARalpha: Mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. *Toxicology,* **246,** 2-8.

Goodwin, B. J., Zuercher, W. J. and Collins, J. L. (2008) Recent advances in liver X receptor biology and chemistry. *Current topics in medicinal chemistry,* **8,** 781-791.

Gould, S. J. and Valle, D. (2000) Peroxisome biogenesis disorders: genetics and cell biology. *Trends Genet,* **16,** 340-345.

Guo, T., Gregg, C., Boukh-Viner, T. et al. (2007) A signal from inside the peroxisome initiates its division by promoting the remodeling of the peroxisomal membrane. *The Journal of cell biology,* **177,** 289-303.

Hogenboom, S., Wanders, R. J. and Waterham, H. R. (2003) Cholesterol biosynthesis is not defective in peroxisome biogenesis defective fibroblasts. *Molecular genetics and metabolism,* **80,** 290-295.

Hu, T., Foxworthy, P., Siesky, A. et al. (2005) Hepatic peroxisomal fatty acid beta-oxidation is regulated by liver X receptor alpha. *Endocrinology,* **146,** 5380-5387.

Jick, H., Zornberg, G. L., Jick, S. S., Seshadri, S. and Drachman, D. A. (2000) Statins and the risk of dementia. *Lancet,* **356,** 1627-1631.

Joyce, C. W., Wagner, E. M., Basso, F. et al. (2006) ABCA1 overexpression in the liver of LDLr-KO mice leads to accumulation of pro-atherogenic lipoproteins and enhanced atherosclerosis. *The Journal of biological chemistry,* **281,** 33053-33065.

Kase, E. T., Thoresen, G. H., Westerlund, S., Hojlund, K., Rustan, A. C. and Gaster, M. (2007) Liver X receptor antagonist reduces lipid formation and increases glucose metabolism in myotubes from lean, obese and type 2 diabetic individuals. *Diabetologia,* **50,** 2171-2180.

Kim, H. J., Fan, X., Gabbi, C., Yakimchuk, K., Parini, P., Warner, M. and Gustafsson, J. A. (2008) Liver X receptor beta (LXRbeta): a link between beta-sitosterol and amyotrophic lateral sclerosis-Parkinson's dementia. *Proceedings of the National Academy of Sciences of the United States of America,* **105,** 2094-2099.

Kim, P. K., Mullen, R. T., Schumann, U. and Lippincott-Schwartz, J. (2006) The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *The Journal of cell biology,* **173,** 521-532.

Kobayashi, S., Tanaka, A. and Fujiki, Y. (2007) Fis1, DLP1, and Pex11p coordinately regulate peroxisome morphogenesis. *Exp Cell Res,* **313,** 1675-1686.

Kotti, T. J., Ramirez, D. M., Pfeiffer, B. E., Huber, K. M. and Russell, D. W. (2006) Brain cholesterol turnover required for geranylgeraniol production and learning in mice. *Proceedings of the National Academy of Sciences of the United States of America,* **103,** 3869-3874.

Kovacs, W. J., Shackelford, J. E., Tape, K. N., Richards, M. J., Faust, P. L., Fliesler, S. J. and Krisans, S. K. (2004) Disturbed cholesterol homeostasis in a peroxisome-deficient PEX2 knockout mouse model. *Molecular and cellular biology,* **24,** 1-13.

Kovacs, W. J., Tape, K. N., Shackelford, J. E., Duan, X., Kasumov, T., Kelleher, J. K., Brunengraber, H. and Krisans, S. K. (2007) Localization of the pre-squalene segment of the isoprenoid biosynthetic pathway in mammalian peroxisomes. *Histochem Cell Biol,* **127,** 273-290.

Lee, S. S., Pineau, T., Drago, J., Lee, E. J., Owens, J. W., Kroetz, D. L., Fernandez-Salguero, P. M., Westphal, H. and Gonzalez, F. J. (1995) Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Molecular and cellular biology,* **15,** 3012-3022.

Lein, E. S., Hawrylycz, M. J., Ao, N. et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature,* **445,** 168-176.

Levison, S. W. and McCarthy, K. D. (1991) *Culturing Nerve Cell*. MIT Press, Cambridge, MA.

Li, X. and Gould, S. J. (2002) PEX11 promotes peroxisome division independently of peroxisome metabolism. *The Journal of cell biology,* **156,** 643-651.

Lund, E. G., Xie, C., Kotti, T., Turley, S. D., Dietschy, J. M. and Russell, D. W. (2003) Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specific mechanism of cholesterol turnover. *The Journal of biological chemistry,* **278,** 22980-22988.

Mauch, D. H., Nagler, K., Schumacher, S., Goritz, C., Muller, E. C., Otto, A. and Pfrieger, F. W. (2001) CNS synaptogenesis promoted by glia-derived cholesterol. *Science (New York, N.Y,* **294,** 1354-1357.

Michalik, L., Desvergne, B., Dreyer, C., Gavillet, M., Laurini, R. N. and Wahli, W. (2002) PPAR expression and function during vertebrate development. *The International journal of developmental biology,* **46,** 105-114.

Mitro, N., Vargas, L., Romeo, R., Koder, A. and Saez, E. (2007) T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. *FEBS letters,* **581,** 1721-1726.

Peet, D. J., Janowski, B. A. and Mangelsdorf, D. J. (1998) The LXRs: a new class of oxysterol receptors. *Current opinion in genetics & development,* **8,** 571-575.

Ramirez, D. M., Andersson, S. and Russell, D. W. (2008) Neuronal expression and subcellular localization of cholesterol 24-hydroxylase in the mouse brain. *The Journal of comparative neurology,* **507,** 1676-1693.

Reddy, J. K. and Chu, R. (1996) Peroxisome proliferator-induced pleiotropic responses: pursuit of a phenomenon. *Annals of the New York Academy of Sciences,* **804,** 176-201.

Repa, J. J., Li, H., Frank-Cannon, T. C., Valasek, M. A., Turley, S. D., Tansey, M. G. and Dietschy, J. M. (2007) Liver X receptor activation enhances cholesterol loss from the brain, decreases neuroinflammation, and increases survival of the NPC1 mouse. *J Neurosci,* **27,** 14470-14480.

Repa, J. J., Liang, G., Ou, J. et al. (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes & development,* **14,** 2819-2830.

Santos, M. J., Quintanilla, R. A., Toro, A., Grandy, R., Dinamarca, M. C., Godoy, J. A. and Inestrosa, N. C. (2005) Peroxisomal proliferation protects from beta-amyloid neurodegeneration. *The Journal of biological chemistry,* **280,** 41057-41068.

Schrader, M., Reuber, B. E., Morrell, J. C., Jimenez-Sanchez, G., Obie, C., Stroh, T. A., Valle, D., Schroer, T. A. and Gould, S. J. (1998) Expression of PEX11beta mediates peroxisome proliferation in the absence of extracellular stimuli. *The Journal of biological chemistry,* **273,** 29607-29614.

Schrader, M. and Yoon, Y. (2007) Mitochondria and peroxisomes: are the 'big brother' and the 'little sister' closer than assumed? *Bioessays,* **29,** 1105-1114.

Schultz, J. R., Tu, H., Luk, A. et al. (2000) Role of LXRs in control of lipogenesis. *Genes & development,* **14,** 2831-2838.

Sinensky, M. (2000) Functional aspects of polyisoprenoid protein substituents: roles in protein-protein interaction and trafficking. *Biochimica et biophysica acta,* **1529,** 203-209.

Singh, I., Pahan, K. and Khan, M. (1998) Lovastatin and sodium phenylacetate normalize the levels of very long chain fatty acids in skin fibroblasts of X-adrenoleukodystrophy. *FEBS letters,* **426,** 342-346.

Soccio, R. E. and Breslow, J. L. (2004) Intracellular cholesterol transport. *Arteriosclerosis, thrombosis, and vascular biology,* **24,** 1150-1160.

South, S. T. and Gould, S. J. (1999) Peroxisome synthesis in the absence of preexisting peroxisomes. *The Journal of cell biology,* **144,** 255-266.

Steffensen, K. R. and Gustafsson, J. A. (2004) Putative metabolic effects of the liver X receptor (LXR). *Diabetes,* **53 Suppl 1,** S36-42.

Steffensen, K. R., Neo, S. Y., Stulnig, T. M., Vega, V. B., Rahman, S. S., Schuster, G. U., Gustafsson, J. A. and Liu, E. T. (2004) Genome-wide expression profiling; a panel of mouse tissues discloses novel biological functions of liver X receptors in adrenals. *Journal of molecular endocrinology,* **33,** 609-622.

Sun, Y., Yao, J., Kim, T. W. and Tall, A. R. (2003) Expression of liver X receptor target genes decreases cellular amyloid beta peptide secretion. *The Journal of biological chemistry,* **278,** 27688-27694.

Titorenko, V. I. and Mullen, R. T. (2006) Peroxisome biogenesis: the peroxisomal endomembrane system and the role of the ER. *The Journal of cell biology,* **174,** 11-17.

Tobin, K. A., Steineger, H. H., Alberti, S., Spydevold, O., Auwerx, J., Gustafsson, J. A. and Nebb, H. I. (2000) Cross-talk between fatty acid and cholesterol metabolism mediated by liver X receptor-alpha. *Molecular endocrinology (Baltimore, Md,* **14,** 741-752.

Vosper, H., Khoudoli, G. A., Graham, T. L. and Palmer, C. N. (2002) Peroxisome proliferator-activated receptor agonists, hyperlipidaemia, and atherosclerosis. *Pharmacology & therapeutics,* **95,** 47-62.

Wanders, R. J. and Waterham, H. R. (2006) Biochemistry of mammalian peroxisomes revisited. *Annual review of biochemistry,* **75,** 295-332.

Wang, Y., Rogers, P. M., Su, C., Varga, G., Stayrook, K. R. and Burris, T. P. (2008) Regulation of cholesterologenesis by the oxysterol receptor, LXRalpha. *The Journal of biological chemistry*.

Wei, H., Kemp, S., McGuinness, M. C., Moser, A. B. and Smith, K. D. (2000) Pharmacological induction of peroxisomes in peroxisome biogenesis disorders. *Annals of neurology,* **47,** 286-296.

Weinhofer, I., Forss-Petter, S., Kunze, M., Zigman, M. and Berger, J. (2005) X-linked adrenoleukodystrophy mice demonstrate abnormalities in cholesterol metabolism. *FEBS letters,* **579,** 5512-5516.

Whitney, K. D., Watson, M. A., Collins, J. L. et al. (2002) Regulation of cholesterol homeostasis by the liver X receptors in the central nervous system. *Molecular endocrinology (Baltimore, Md,* **16,** 1378-1385.

Won, J. S., Im, Y. B., Khan, M., Contreras, M., Singh, A. K. and Singh, I. (2008) Lovastatin inhibits amyloid precursor protein (APP) beta-cleavage through reduction of APP distribution in Lubrol WX extractable low density lipid rafts. *Journal of neurochemistry,* **105,** 1536-1549.

Wong, J., Quinn, C. M., Guillemin, G. and Brown, A. J. (2007) Primary human astrocytes produce 24(S),25-epoxycholesterol with implications for brain cholesterol homeostasis. *Journal of neurochemistry,* **103,** 1764-1773.

Zhang, Y. and Mangelsdorf, D. J. (2002) LuXuRies of lipid homeostasis: the unity of nuclear hormone receptors, transcription regulation, and cholesterol sensing. *Molecular interventions,* **2,** 78-87.

Figure legends

Fig. 1 Differential effects of PPAR α agonists on FAO hepatocyte-like cells versus rat astrocytes. FAO cells (left) or astrocytes (right) were plated onto coverslips and then treated with 10 μm WY14643 or 6 nM GW7647 for 48 hr; then cells were fixed and peroxisomes quantified. (a-c) examples of typical peroxisome morphologies in FAO cells (a) control (b) WY14643 and (c) GW 76473. (d-f) quantification of drug-induced changes in peroxisome size and number. (g-i) examples of typical peroxisome morphologies in astrocytes (g) control, (h) WY14643 and (i) GW 76473. Bar indicates 1 μm. (j-l) quantification of peroxisome size and number in astrocytes. PPAR α agonists had no detectable effect on peroxisomes in astrocytes. Graphs depict mean +/- S.D. *p <0.05 versus control; **p < 0.01 versus control.

Fig. 2 LXR receptors regulate peroxisome number and area in astrocytes. Rat astrocytes were plated on coverslips and then treated with 250 nM T09 and/or 10 μM 22(S)-HC before fixation and morphological assay of peroxisomes. (a) time course of the apposing effects of the agonist (T09, open square) versus antagonist (22(S)-HC, closed circle) or both drugs together (bolded x). (b-e) representative images of peroxisomes used for quantification at low power (top of each panel) or as high-power montages (bottom of each panel) for each drug treatment. Bar in top panels is 10 μm; bar in lower panel montages is 1 μm. (f-h), quantification of LXR drug-induced changes in peroxisome size and number. T09 increased density of peroxisomes. Graphs depict mean +/- S.D . *p < 0.05 versus control; **p < 0.01 versus control.

Fig. 3 LXR drugs do not affect mitochondrial abundance. Astrocytes were treated with drugs and fixed as described for Fig. 2. Mitochondria were visualized with an antibody against ATP synthase and area in the cell occupied by mitochondria was measured. No significant differences in mitochondrial area were caused by the drugs.

Fig. 4 Lovastatin induces peroxisome proliferation. Rat astrocytes were plated on coverslips and then treated with 50 nM lovastatin and/or 10 μM 22(S)-HC for two days before fixation and morphological assay of peroxisomes. (a) time course of lovastain-induced proliferation (open squares) versus antagonist 22(S)-HC (closed circles) or both drugs together (filled squares). (b-d) representative images of peroxisomes used for quantification at low power (top of each panel) or as high-power montages (bottom of each panel) for each drug treatment. Bar in top panels is 10 μm; bar in lower panel montages is 1 μm. (e-g) quantification of lovastatin-induced changes in peroxisome size and number. Lovastatin increased the density of peroxisomes, and 22(S)-HC prevented the increase. Graphs depict mean +/- S.D . *p < 0.05 versus control; **p < 0.01 versus control.

Fig. 5 LXR antagonist and lovastatin treatments in FAO cells regulate peroxisome abundance. FAO cells were treated with T09, lovastatin, or 22(S)-HC for 48 hr. Cells were then fixed and peroxisomes quantified. Graphs depict mean +/- S.D . *p < 0.05 versus control; **p < 0.01 versus control.

Fig 6 LXR agonist T09 induces ABCA1 protein and activity. (a) Western blot analysis of astrocyte cell lysates 48 hr after treatment with 22(S)-HC, T09 and lovastatin. T09 led to a 5-fold increase over control, while levels of actin did not change. (b-e) following drug treatments as above, the cellular localization of cholesterol was visualized with filipin in the absence (b, d) or presence (c, e) of ApoA1. (b) control; (c) control + ApoA1; (d) T09; (e) T09 + ApoA1. Arrows in (d) indicate increased plasma membrane cholesterol after T09. Arrows in (e) indicate reduced plasma membrane cholesterol when ApoA1 is included in the growth medium of the T09-treated astrocytes. Bar indicates 10 μm. Graphs depict mean +/- S.D **$p < 0.01$ versus control.

Fig. 7 LXR agonist T09 does affect levels of 4 different proteins that potentially could participate in the control of peroxisome number. Astrocytes were treated with 10 μM 22(S)-HC, 250 nM T09, 50 nM lovastatin, or 5 mM 4PB for 48 hours and then cells were scraped and analyzed by Western Blot. (a) Blots of astrocyte cell lysates (10 μg/ lane). (b) 4-PBA, but not other treatments, caused a 3-fold increase in expression of ABCD2. (c, d, e) No significant changes were seen in PMP70 (c), Acox (d) or DLP1 (e). Graphs depict mean +/- S.D **$p < 0.01$ versus control.

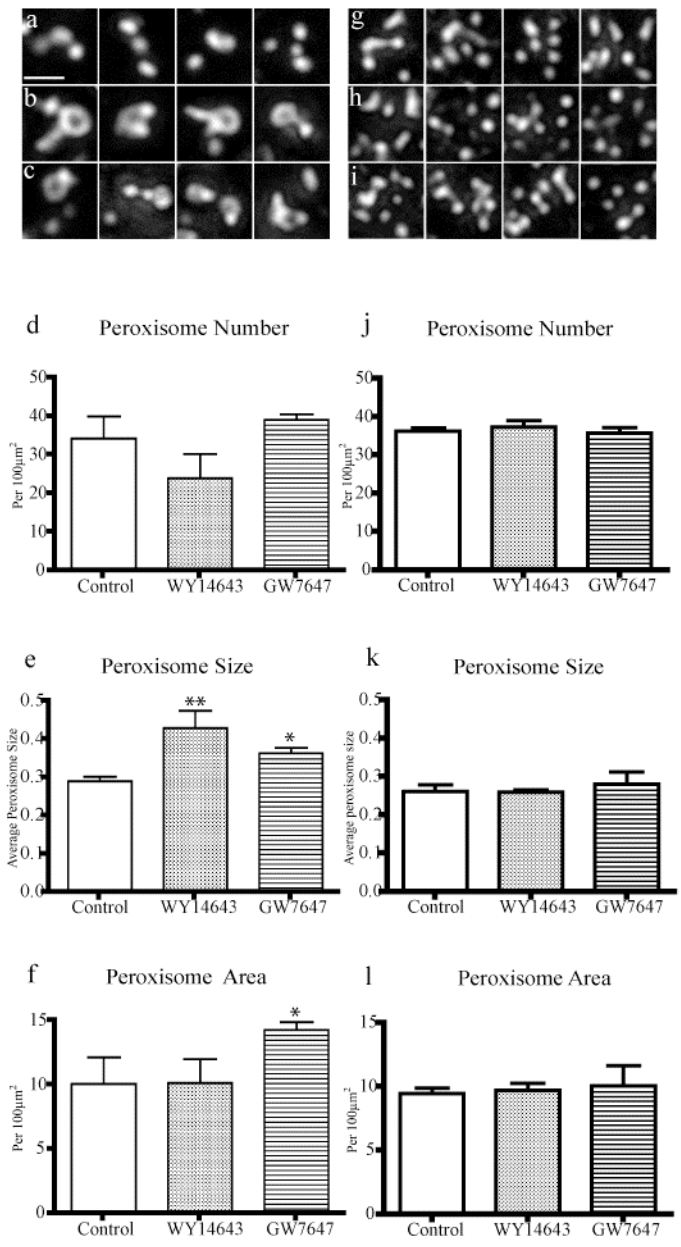

a
b
c
g
h
i
d
Peroxisome Number
Per 100μm²
Control
WY14643
GW7647
j
Peroxisome Number
Per 100μm²
Control
WY14643
GW7647
e
Peroxisome Size
Average Peroxisome Size
**
*
Control
WY14643
GW7647
k
Peroxisome Size
Average peroxisome size
Control
WY14643
GW7647
f
Peroxisome Area
Per 100μm²
*
Control
WY14643
GW7647
l
Peroxisome Area
Per 100μm²
Control
WY14643
GW7647

Figure 1

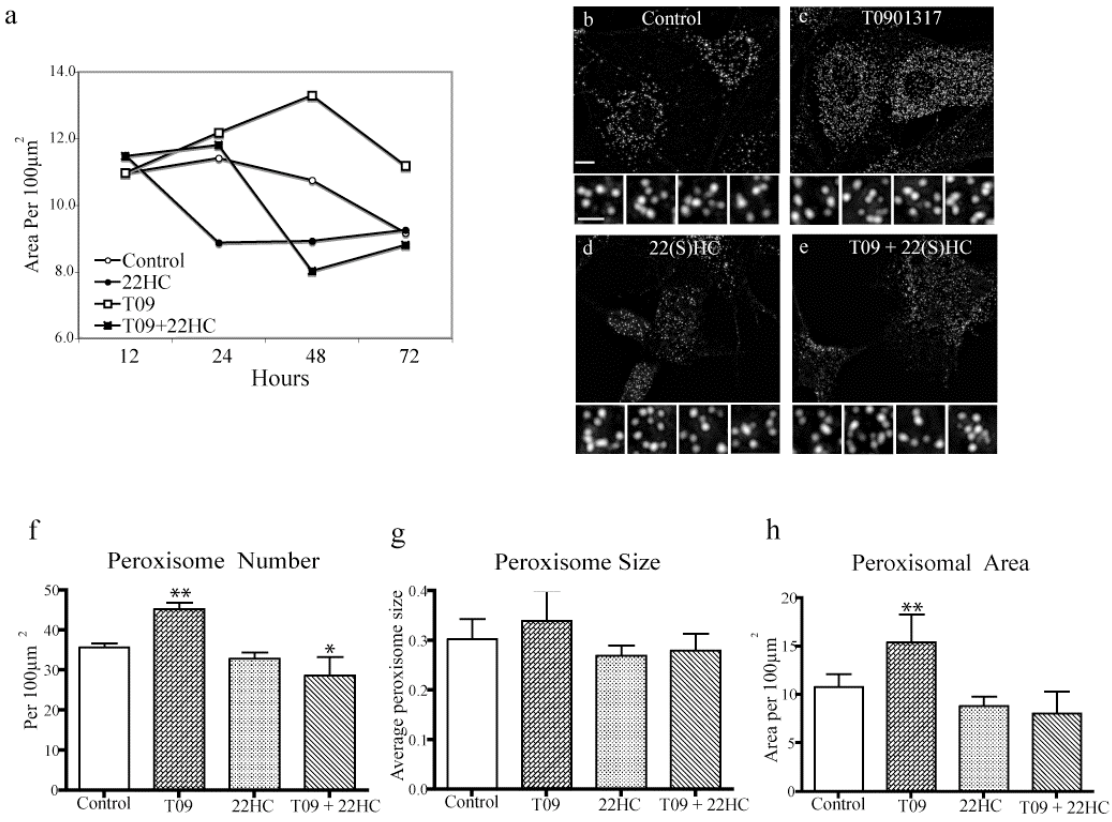

a
Area Per 100μm 2
14.0
12.0
10.0
8.0
6.0
Control
22HC
T09
T09+22HC
12
24
48
72
Hours
b Control
c T0901317
d 22(S)HC
e T09 + 22(S)HC
f
Peroxisome Number
Per 100μm 2
50
40
30
20
10
0
**
*
Control
T09
22HC
T09 + 22HC
g
Peroxisome Size
Average peroxisome size
0.4
0.3
0.2
0.1
0.0
Control
T09
22HC
T09 + 22HC
h
Peroxisomal Area
Area per 100μm 2
20
15
10
5
0
**
Control
T09
22HC
T09 + 22HC

Figure 2

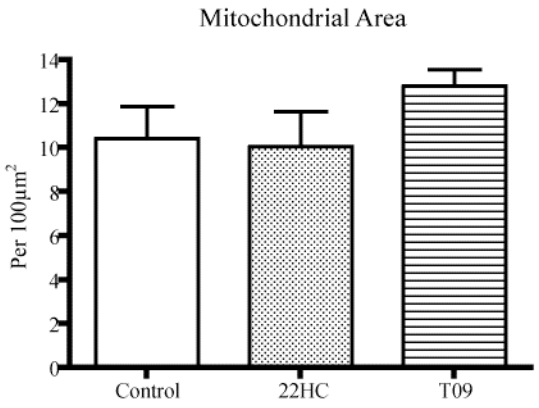

Mitochondrial Area
Per 100μm²
14
12
10
8
6
4
2
0
Control
22HC
T09

Figure 3

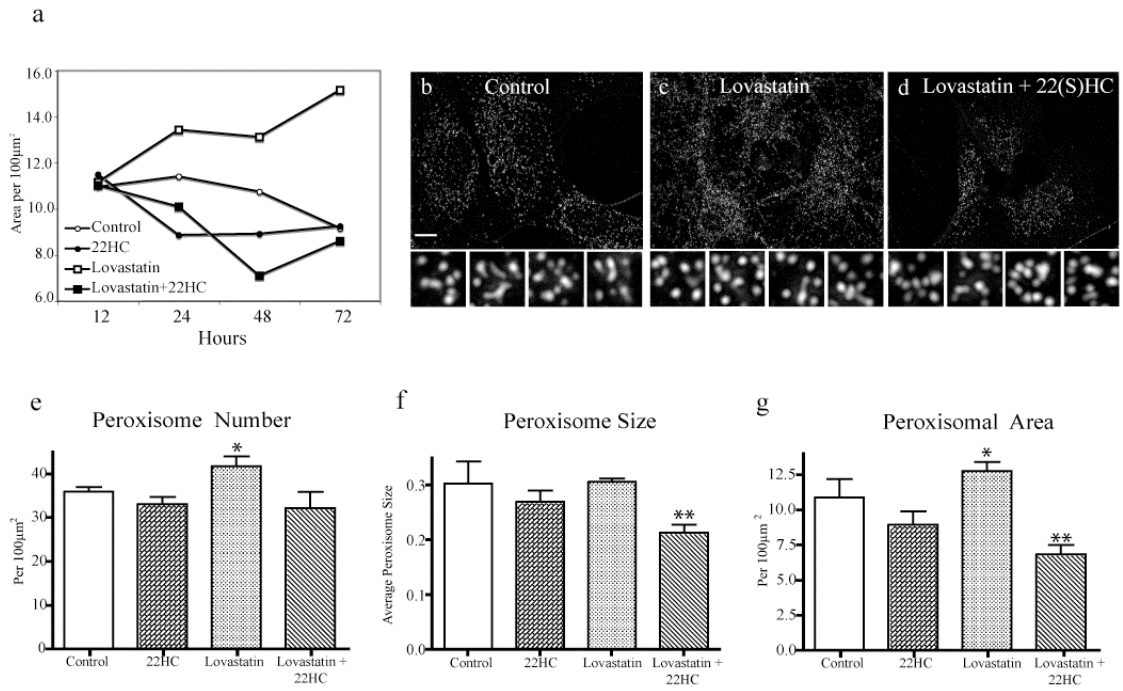

a
Area per 100µm²
16.0
14.0
12.0
10.0
8.0
6.0
Control
22HC
Lovastatin
Lovastatin+22HC
12
24
48
72
Hours
b Control
c Lovastatin
d Lovastatin + 22(S)HC
e
Peroxisome Number
Per 100µm²
40
30
20
10
0
*
Control
22HC
Lovastatin
Lovastatin + 22HC
f
Peroxisome Size
Average Peroxisome Size
0.3
0.2
0.1
0.0
**
Control
22HC
Lovastatin
Lovastatin + 22HC
g
Peroxisomal Area
Per 100µm²
12.5
10.0
7.5
5.0
2.5
0.0
*
**
Control
22HC
Lovastatin
Lovastatin + 22HC

Figure 4

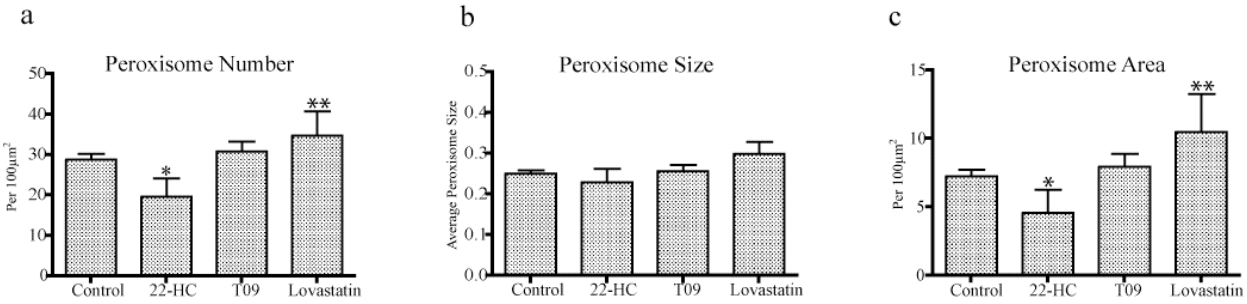

a
Peroxisome Number
Per 100μm²
50
40
30
20
10
0
*
**
Control
22-HC
T09
Lovastatin
b
Peroxisome Size
Average Peroxisome Size
0.5
0.4
0.3
0.2
0.1
0.0
Control
22-HC
T09
Lovastatin
c
Peroxisome Area
Per 100μm²
15
10
5
0
*
**
Control
22-HC
T09
Lovastatin

Figure 5

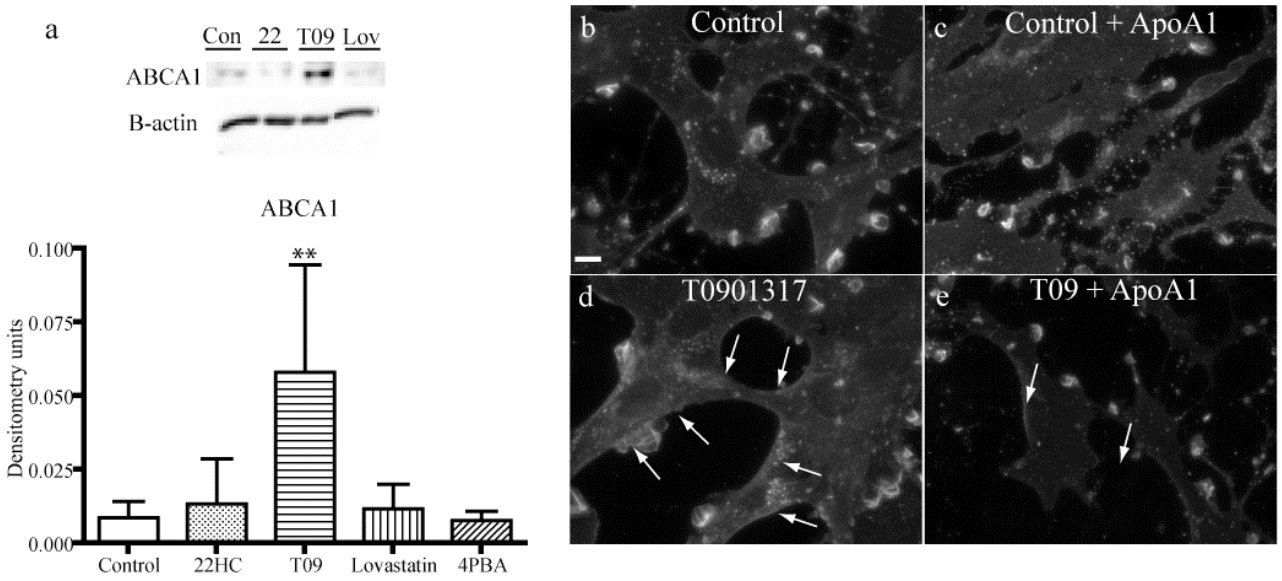

a
Con 22 T09 Lov
ABCA1
B-actin
ABCA1
**
Densitometry units
0.100
0.075
0.050
0.025
0.000
Control
22HC
T09
Lovastatin
4PBA
b Control
c Control + ApoA1
d T0901317
e T09 + ApoA1

Figure 6

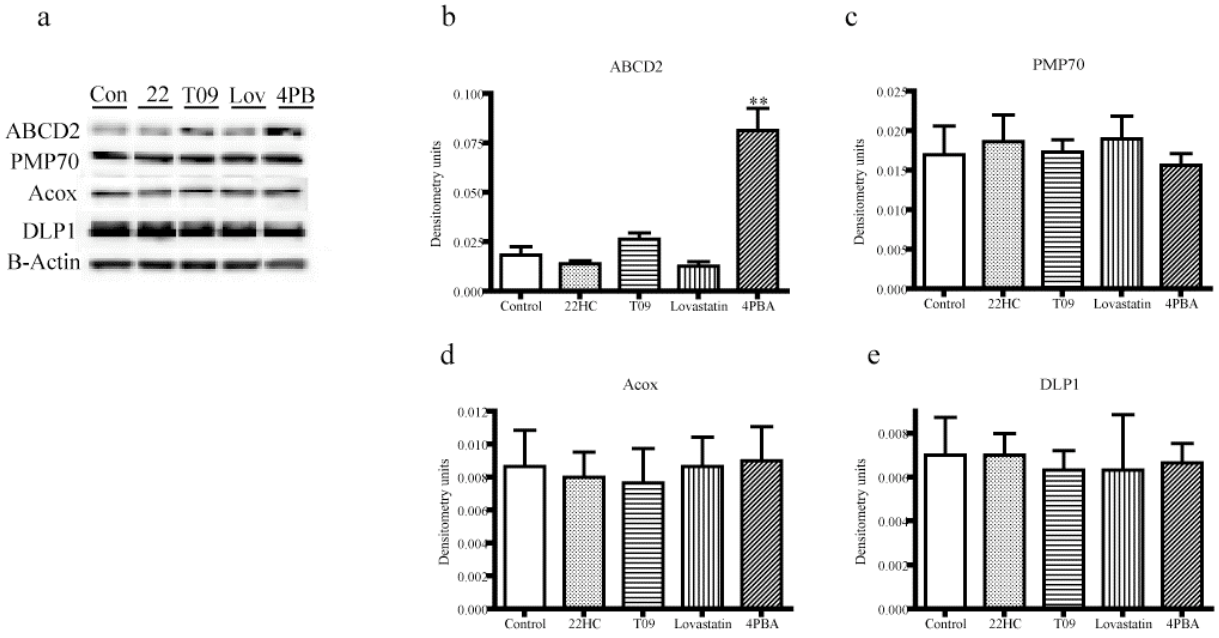

a
Con
22
T09
Lov
4PB
ABCD2
PMP70
Acox
DLP1
B-Actin
b
ABCD2
Densitometry units
0.100
0.075
0.050
0.025
0.000
**
Control
22HC
T09
Lovastatin
4PBA
c
PMP70
Densitometry units
0.025
0.020
0.015
0.010
0.005
0.000
Control
22HC
T09
Lovastatin
4PBA
d
Acox
Densitometry units
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Control
22HC
T09
Lovastatin
4PBA
e
DLP1
Densitometry units
0.008
0.006
0.004
0.002
0.000
Control
22HC
T09
Lovastatin
4PBA

Figure 7

Chapter 3: Implications and Future Research

Determining the molecular mechanisms that control peroxisome number in brain.

Our studies uncovered a novel pharmacological means of controlling peroxisome number in astrocytes. As the LXR is a ligand-regulated nuclear receptor, the underlying mechanisms may involve changes in gene transcription. However, the precise gene targets that account for our observations remain unclear. Previous studies indicate that two main biosynthetic pathways control peroxisome abundance in cells (see Chapter 1, Figure 1). Changes in expression of the proteins that control the fission pathway appear to account for all previously described reports of peroxisome proliferation (Table 1). In yeast, peroxisome abundance is tightly linked to the type of energy substrate on which yeast grow. For example, yeast peroxisomes can be made to proliferate by growing the yeast on the fatty acid oleate (Marshall *et al.* 1995, Erdmann & Blobel 1995). This occurs through the activation of Pip2p and Oaf1p transcription factors that regulate Pex11 in *S. cerevisiae* (Rottensteiner *et al.* 1997, Gurvitz *et al.* 2001). Yeast grown on high-glucose medium on the other hand contain very few peroxisomes. This is not surprising as, in yeast, all fatty acid oxidation occurs within peroxisomes while in mammals, fatty acid oxidation within peroxisomes is restricted to long-chain and VLCFAs.

In rodent liver, peroxisomes can be induced to proliferate by the fibrate drugs, which bind and activate PPARα and cause transcription of proteins involved in peroxisome fission. Surprisingly, in man, PPARα does not appear to respond to fibrates in the manner as the homolog from rodents (Gonzalez & Shah 2008). Peroxisome

proliferation in humans has been achieved with the drug 4-PBA (Wei *et al.* 2000), an atypical peroxisome proliferator in that its actions are not through PPARα but rather as a histone deacetylase inhibitor that leads to an increase in the transcription of Pex11. In all three cases, peroxisomal proliferation in response to an environmental change is through the fission pathway via Pex11.

Table 1. Known pharmaceutical inducers of peroxisomes

	Yeast	Rodent	Human
Inducer	Fatty acids; oleate	Fibrates; PPARα agonists	4PBA
Target	Plp2-Paf1p; inhibition of HDACs	PPARα	HDAC inhibitor of the Pex11 promoter region
Gene	Pex11p	Pex11	Pex11
Pathway	Fission	Fission	Fission

As there are currently no commercial antibodies to Pex11, we were unable to test if a Pex11-dependent pathway was also responsible for the changes in peroxisomal abundance in our model. Furthermore, examination of the expression pattern of Pex11 isoforms in brain indicates that Pex11, although present in brain, is not particularly abundant (Figure 1). An alternative mechanism to explain our results is that the peroxisome proliferation occurs via the *de novo* pathway, through a Pex16-dependent mechanism. Previous microarray studies have shown that LXR agonists do indeed induce Pex16. Wild type and LXR knockout mice were treated with T09 and total RNA was extracted. T09 treatment in the wild type but not knockout groups showed an up-regulation of Pex16 (Steffensen et al. 2004). Pex16 is known to localize in sub-compartments of the ER and function in the recruitment of other biogenesis machinery

(Pex3, PMP34, and Pex19). Moreover, Pex16, by contrast to Pex11 is highly abundant in all brain areas (Figure 1). To distinguish between the two most likely pathways, we plan to measure levels of their mRNAs with quantitative PCR following the drug treatments that regulate peroxisome abundance in our model. This would indicate whether the peroxisomal proliferation could be mediated through increased Pex16 transcription. If on the other hand Pex16 levels remain unchanged in our model, the possible explanations are that LXR drugs affect Pex16 activity via their effects on cholesterol metabolism—or that Pex16 is not involved whatsoever.

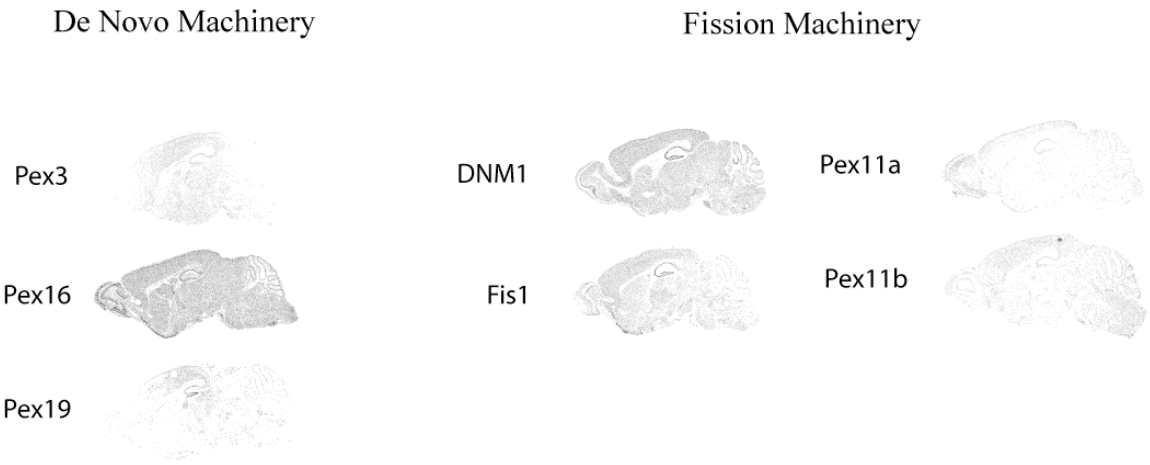


Figure 1. Expression analysis of peroxisome biogenesis pathways.

Photoactivation/pulse-chase assays conducted by the Lippincott-Schwartz laboratory at the NIH have visualized the movement of Pex16 from the ER to nascent peroxisomes in COS7 cells (Kim et al. 2006). In future studies the Schell laboratory plans to collaborate with this group to test the hypothesis that the LXR-mediated

peroxisome proliferation that we have discovered is operating via Pex16-dependent mechanisms discovered by their laboratory. To accomplish this, our plan is to first grow brain cells in the presence of 22(S)-HC in order to reduce peroxisome numbers. At that point, cells will be transfected with Pex16 fused to a photo-switchable fluorescent protein (Kim et al. 2006). The cells will then be washed out of 22(S)-HC and exposed to TO9, the LXR agonist. This will maximize our ability to observe *de novo* synthesis of peroxisomes—a sort of pulse/chase approach to observe the biogenesis of peroxisomes in living cells. Since the Schell laboratory is expert in growing and transfecting neurons (Schell *et al.* 2001), and levels of Pex16 are very high in this cell type, the aim will be to accomplish these studies in hippocampal neurons. However, before such an approach can be attempted, it will be essential to establish that the LXR mediated proliferation that we have discovered in astrocytes also occurs in neurons. These studies are currently underway in the Schell laboratory.

In addition to using the photo-activated form of GFP used in the previous study (Kim et al. 2006), we will also fuse Pex16 to a protein called Dendra2, which shifts from green to red fluorescence emission when activated by 405 nm wavelength light (Chudakov *et al.* 2007). By using this protein we will simultaneously observe the steady-state pool of Pex16 along with the nascent pool. This approach will determine whether the Pex16-driven *de novo* pathway is favored following application of LXR agonists, and may also allow the first visualization of peroxisomal birthing event.

Further development of brain-selective peroxisome induction

As mentioned above, previous attempts to capitalize on peroxisome proliferation to treat human disease has focused mainly on the drug 4-PBA. This compound is already in clinical use for treating disorders of the urea cycle (Burlina *et al.* 2001), but has also been examined as a treatment for X-ALD as an inducer of ABCD2 expression. However, due to its rapid metabolism (1-2 hr) and low affinity (IC50 in the millimolar range), it is unfavorable for patients. Doses consist of 20g, equaling 40 pills daily (Kemp & Wanders 2007). Lovastatin, due to its normalization of VLCFA in the plasma of patients with X-ALD (Pai *et al.* 2000), is undergoing clinical trials; however, side effects have been problematic in that it worsens the blood lipid profiles for other fatty acids (Engelen *et al.* 2008). Peroxisomal proliferation in either case was not examined, although theoretically the increase in VLCFA oxidation could be explained by subsequent increases in peroxisomal abundance by lovastatin.

The LXR class of drugs is beginning to appear in clinical trials for cholesterol control, so our discovery offers the potential for rapid translation to the clinic should it continue to show promise. Pharmacological manipulation of cholesterol efflux from the brain is under investigation for numerous neurological disorders including, but not limited to, AD and NPC. In AD, high serum cholesterol is considered an independent risk factor (Notkola *et al.* 1998). In 2000, the role of statins in the pathogenesis of AD and other forms of dementia was examined; authors reported a substantially lowered risk of developing dementia after statin treatment (Jick *et al.* 2000). Similarly, T09 was shown to

lower β-amyloid levels and improve memory in a mouse model of AD (Riddell *et al.* 2007). Our studies raise the possibility that it may be possible to selectively proliferate peroxisomes as therapies for neurodegenerative disorders. This claim is strengthened by the lack of induced peroxisomal abundance in hepatocytes treated with LXR agonists.

One explanation for this may lie with the isoform of LXR receptor present. The distribution of LXRα and β vary greatly between tissues; LXRα which is highly abundant is liver is barely present in the brain, whereas LXRβ is expressed strongly (Zhang & Mangelsdorf 2002). While the two variants are almost identical in structure, it may be possible that in astrocytes, LXRβ induces different target genes. This is not outside the realm of possibility, as it has already been shown that the upregulation of lipoprotein lipase (rate-limiting enzyme in triglyceride hydrolysis) by LXR ligands is due to the actions of LXRα and not β in the liver (Zhang *et al.* 2001). To this end, new compounds have been created which have more isoform-selective properties. N-acylthiadiazoline is being examined for its role as a potential LXRβ-selective agonist. The EC50 of this drug for LXRβ is 63nM with 98% efficacy and for LXRα an EC50 of 350nM with 41% efficacy (Molteni *et al.* 2007). It has not, as of yet, been fully characterized, but offers hope that further improvements in isoform selectivity are on the horizon.

Since most of the known side effects of the current generation of LXR agonists involve unwanted changes in blood lipid profiles, another strategy for improving selectivity of LXR agonists involves selective drug targeting to the CNS. In general, lipophilic molecules effectively cross the blood-brain-barrier. Since LXR is a nuclear receptor for lipophilic compounds, it is not unreasonable to envision further development

of custom LXR agonists with greater selectivity for brain. This type of modulation has already proven beneficial in other systems; for example, in tissue selective estrogen receptor modulators, such as tamoxifen, used to treat breast cancer (Sengupta & Jordan 2008). An attractive third possibility involves the creation of an LXR-selective prodrug. In this scenario, one would take advantage of the brain selective cytochrome P450 46A1, which produces 24-OH cholesterol. This cytochrome shows a highly restricted localization to brain. A prodrug with a cholesterol-like structure that was a substrate for CYP46A1 could potentially be converted to a highly brain-selective LXR agonist, thus eliminating most or all of the side effects in peripheral tissue.

Therapies targeted to nuclear receptors in the CNS.

The LXR is only one of a large class of potential drug targets aimed at selectively regulating transcriptional changes in brain. The nuclear receptor superfamily consists of 48 gene products. Each receptor presumably responds to an endogenous lipophilic molecule present in normal brain. Moreover, many of these receptors occur as isoforms, which make feasible the development of brain-preferring ligands. The generally lipophilic nature of nuclear receptor ligands makes the challenges of targeting to the CNS more surmountable than for most other drug classes (such as drugs designed to mimic the highly charged nature of amino acid neurotransmitters).

The publication of the Allen Brain Atlas offers an opportunity to jump-start research in the area of nuclear receptors in neuropharmacology. Figure 2 is an example of the RAR-related orphan receptor alpha, the mutation of which creates the *staggerer* mouse presentation. As expected, the highest levels of expression are localized to the

cerebellum and thalamus. Such a selective localization fits nicely to the known motor phenotypes observed in *staggerer*. The endogenous ligand for RAR-related orphan receptor alpha is unknown.

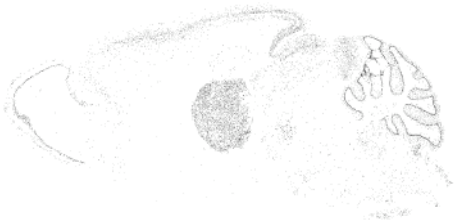
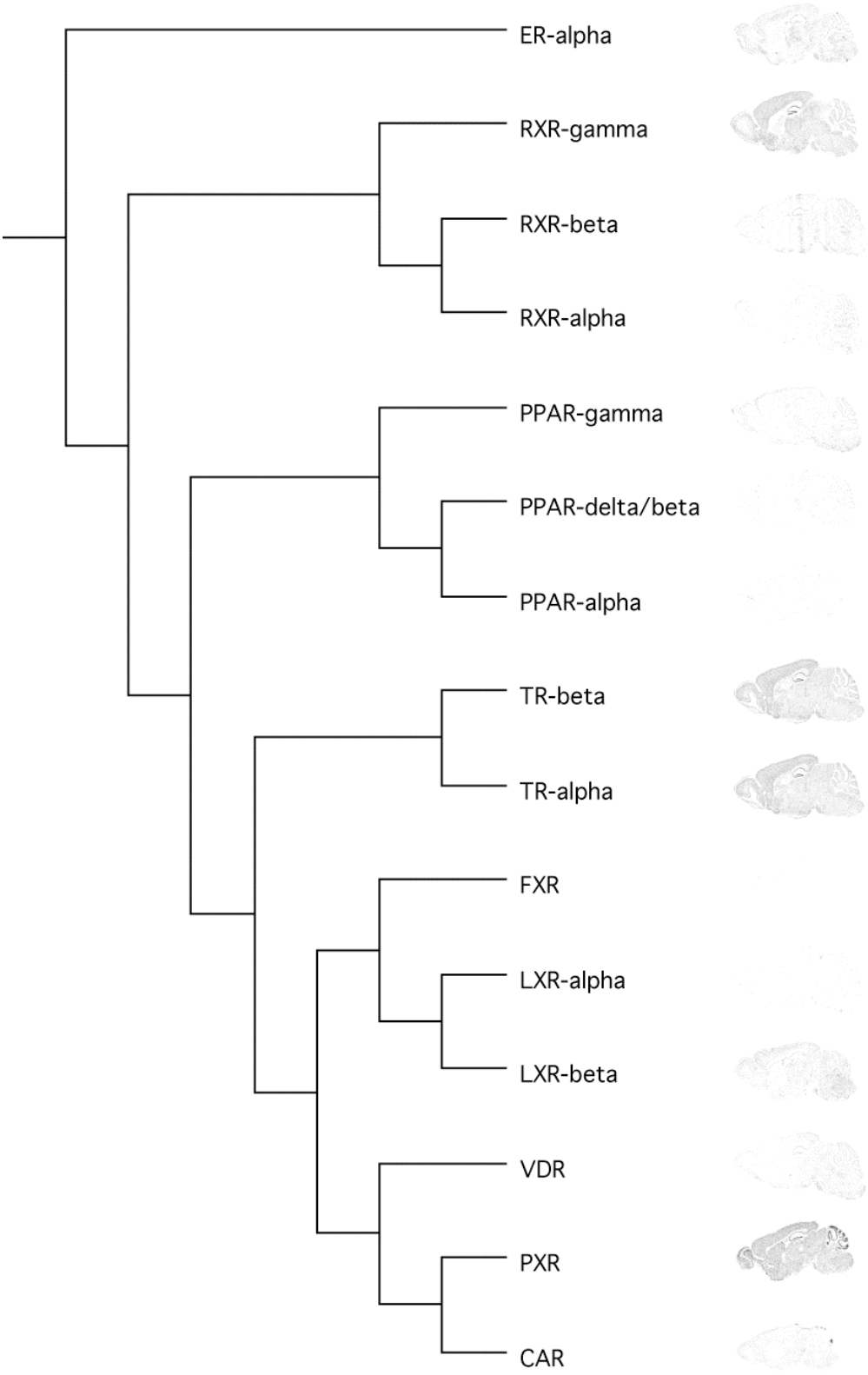


Figure 2. The gene expression of RAR-related orphan receptor alpha, the *staggerer* mouse.

Figure 3 depicts the major nuclear receptors involved in our research, along with its close relatives. What is immediately apparent is the highly heterogeneous expression and localization of each receptor. Take, for example, the differences in expression between the isoforms of LXR. The binding partner for LXR is the RXR, and based on the expression analysis shown below, exploring the dimerization with RXRγ may provide a deeper understanding of the LXR-based effect we perceived. The pregnane X receptor, PXR, showed surprisingly high expression throughout the brain with increased expression seen in the cerebellum. PXR agonists have been explored in the treatment of NPC; through upregulation of PXR targets genes, functional and survival benefits are seen (Langmade *et al.* 2006). However, the PXR receptor can be activated by numerous ligands, as its binding region is large and flexible (Watkins *et al.* 2001). Indeed, activation has been reported via the application of LXR agonists, such as T09 (Mitro *et al.* 2007). To rule out involvement of PXR in our effect, identification and application of a PXR selective antagonist will need to be used.

Figure 3. Nuclear receptors with brain expression analysis



Summary

Our investigation of a novel form of pharmaceutically induced peroxisome proliferation in astrocytes has revealed the possibility of new therapies for treating disorders in peroxisome biogenesis and metabolism. We have also provided a new line of evidence to suggest a relationship between cholesterol homeostasis and peroxisome abundance that may have relevance to therapies for AD and NPC, which are based on cholesterol control in brain cells. We continue to pursue the mechanisms underlying our novel discovery. The widespread expression of LXR in brain has focused our attention son the large number of nuclear receptors present in brain and their discrete localizations. Thus, our study points to the promise of further drug development based on molecules targeted to the more than 20 different nuclear receptor subtypes expressed in brain.

REFERENCE LIST

Anderson, S. P., Dunn, C., Laughter, A. et al. (2004) Overlapping transcriptional programs regulated by the nuclear receptors peroxisome proliferator-activated receptor alpha, retinoid X receptor, and liver X receptor in mouse liver. *Molecular pharmacology,* **66,** 1440-1452.

Andersson, S., Gustafsson, N., Warner, M. and Gustafsson, J. A. (2005) Inactivation of liver X receptor beta leads to adult-onset motor neuron degeneration in male mice. *Proceedings of the National Academy of Sciences of the United States of America,* **102,** 3857-3862.

Ando, S., Tanaka, Y., Toyoda, Y. and Kon, K. (2003) Turnover of myelin lipids in aging brain. *Neurochemical research,* **28,** 5-13.

Bellu, A. R., Komori, M., van der Klei, I. J., Kiel, J. A. and Veenhuis, M. (2001a) Peroxisome biogenesis and selective degradation converge at Pex14p. *The Journal of biological chemistry,* **276,** 44570-44574.

Bellu, A. R., Kram, A. M., Kiel, J. A., Veenhuis, M. and van der Klei, I. J. (2001b) Glucose-induced and nitrogen-starvation-induced peroxisome degradation are distinct processes in Hansenula polymorpha that involve both common and unique genes. *FEMS yeast research,* **1,** 23-31.

Bellu, A. R., Salomons, F. A., Kiel, J. A., Veenhuis, M. and Van Der Klei, I. J. (2002) Removal of Pex3p is an important initial stage in selective peroxisome degradation in Hansenula polymorpha. *The Journal of biological chemistry,* **277,** 42875-42880.

Berger, J. P., Akiyama, T. E. and Meinke, P. T. (2005) PPARs: therapeutic targets for metabolic disease. *Trends in pharmacological sciences,* **26,** 244-251.

Bjorkhem, I. and Meaney, S. (2004) Brain cholesterol: long secret life behind a barrier. *Arteriosclerosis, thrombosis, and vascular biology,* **24,** 806-815.

Boukh-Viner, T. and Titorenko, V. I. (2006) Lipids and lipid domains in the peroxisomal membrane of the yeast Yarrowia lipolytica. *Biochimica et biophysica acta,* **1763,** 1688-1696.

Bretillon, L., Lutjohann, D., Stahle, L., Widhe, T., Bindl, L., Eggertsen, G., Diczfalusy, U. and Bjorkhem, I. (2000) Plasma levels of 24S-hydroxycholesterol reflect the balance between cerebral production and hepatic metabolism and are inversely related to body surface. *Journal of lipid research,* **41,** 840-845.

Burlina, A. B., Ogier, H., Korall, H. and Trefz, F. K. (2001) Long-term treatment with sodium phenylbutyrate in ornithine transcarbamylase-deficient patients. *Molecular genetics and metabolism,* **72,** 351-355.

Chang, C. C., South, S., Warren, D., Jones, J., Moser, A. B., Moser, H. W. and Gould, S. J. (1999) Metabolic control of peroxisome abundance. *Journal of cell science,* **112 (Pt 10),** 1579-1590.

Cheung, C., Akiyama, T. E., Ward, J. M., Nicol, C. J., Feigenbaum, L., Vinson, C. and Gonzalez, F. J. (2004) Diminished hepatocellular proliferation in mice humanized for the nuclear receptor peroxisome proliferator-activated receptor alpha. *Cancer research,* **64,** 3849-3854.

Chudakov, D. M., Lukyanov, S. and Lukyanov, K. A. (2007) Using photoactivatable fluorescent protein Dendra2 to track protein movement. *BioTechniques,* **42,** 553, 555, 557 passim.

de Vries, B., Todde, V., Stevens, P., Salomons, F., van der Klei, I. J. and Veenhuis, M. (2006) Pex14p is not required for N-starvation induced microautophagy and in catalytic amounts for macropexophagy in Hansenula polymorpha. *Autophagy,* **2,** 183-188.

Dietschy, J. M. and Turley, S. D. (2001) Cholesterol metabolism in the brain. *Current opinion in lipidology,* **12,** 105-112.

Dietschy, J. M. and Turley, S. D. (2004) Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. *Journal of lipid research,* **45,** 1375-1397.

Eckert, G. P., Vardanian, L., Rebeck, G. W. and Burns, M. P. (2007) Regulation of central nervous system cholesterol homeostasis by the liver X receptor agonist TO-901317. *Neuroscience letters,* **423,** 47-52.

Engelen, M., Ofman, R., Mooijer, P. A., Poll-The, B. T., Wanders, R. J. and Kemp, S. (2008) Cholesterol-deprivation increases mono-unsaturated very long-chain fatty acids in skin fibroblasts from patients with X-linked adrenoleukodystrophy. *Biochimica et biophysica acta,* **1781,** 105-111.

Erdmann, R. and Blobel, G. (1995) Giant peroxisomes in oleic acid-induced Saccharomyces cerevisiae lacking the peroxisomal membrane protein Pmp27p. *The Journal of cell biology,* **128,** 509-523.

Fagarasanu, A., Fagarasanu, M. and Rachubinski, R. A. (2007) Maintaining peroxisome populations: a story of division and inheritance. *Annu Rev Cell Dev Biol,* **23,** 321-344.

Fourcade, S., Savary, S., Albet, S. et al. (2001) Fibrate induction of the adrenoleukodystrophy-related gene (ABCD2): promoter analysis and role of the peroxisome proliferator-activated receptor PPARalpha. *European journal of biochemistry / FEBS,* **268,** 3490-3500.

Gelissen, I. C., Harris, M., Rye, K. A. et al. (2006) ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. *Arteriosclerosis, thrombosis, and vascular biology,* **26,** 534-540.

Gimpl, G. and Gehrig-Burger, K. (2007) Cholesterol reporter molecules. *Biosci Rep,* **27,** 335-358.

Golomb, B. A. (2005) Implications of statin adverse effects in the elderly. *Expert Opin Drug Saf,* **4,** 389-397.

Gondcaille, C., Depreter, M., Fourcade, S. et al. (2005) Phenylbutyrate up-regulates the adrenoleukodystrophy-related gene as a nonclassical peroxisome proliferator. *The Journal of cell biology,* **169,** 93-104.

Gonzalez, F. J. and Shah, Y. M. (2008) PPARalpha: Mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. *Toxicology,* **246,** 2-8.

Goodwin, B. J., Zuercher, W. J. and Collins, J. L. (2008) Recent advances in liver X receptor biology and chemistry. *Current topics in medicinal chemistry,* **8,** 781-791.

Gould, S. J. and Valle, D. (2000) Peroxisome biogenesis disorders: genetics and cell biology. *Trends Genet,* **16,** 340-345.

Gurvitz, A., Hiltunen, J. K., Erdmann, R., Hamilton, B., Hartig, A., Ruis, H. and Rottensteiner, H. (2001) Saccharomyces cerevisiae Adr1p governs fatty acid beta-oxidation and peroxisome proliferation by regulating POX1 and PEX11. *The Journal of biological chemistry,* **276,** 31825-31830.

Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P. and Tabak, H. F. (2005) Contribution of the endoplasmic reticulum to peroxisome formation. *Cell,* **122,** 85-95.

Hogenboom, S., Tuyp, J. J., Espeel, M., Koster, J., Wanders, R. J. and Waterham, H. R. (2004a) Mevalonate kinase is a cytosolic enzyme in humans. *Journal of cell science,* **117,** 631-639.

Hogenboom, S., Tuyp, J. J., Espeel, M., Koster, J., Wanders, R. J. and Waterham, H. R. (2004b) Phosphomevalonate kinase is a cytosolic protein in humans. *Journal of lipid research,* **45,** 697-705.

Honsho, M., Tamura, S., Shimozawa, N., Suzuki, Y., Kondo, N. and Fujiki, Y. (1998) Mutation in PEX16 is causal in the peroxisome-deficient Zellweger syndrome of complementation group D. *American journal of human genetics,* **63,** 1622-1630.

Ishibashi, S., Herz, J., Maeda, N., Goldstein, J. L. and Brown, M. S. (1994) The two-receptor model of lipoprotein clearance: tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proceedings of the National Academy of Sciences of the United States of America,* **91,** 4431-4435.

Janowski, B. A., Willy, P. J., Devi, T. R., Falck, J. R. and Mangelsdorf, D. J. (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature,* **383,** 728-731.

Jick, H., Zornberg, G. L., Jick, S. S., Seshadri, S. and Drachman, D. A. (2000) Statins and the risk of dementia. *Lancet,* **356,** 1627-1631.

Keller, G. A., Barton, M. C., Shapiro, D. J. and Singer, S. J. (1985) 3-Hydroxy-3-methylglutaryl-coenzyme A reductase is present in peroxisomes in normal rat liver cells. *Proceedings of the National Academy of Sciences of the United States of America,* **82,** 770-774.

Kemp, S. and Wanders, R. J. (2007) X-linked adrenoleukodystrophy: very long-chain fatty acid metabolism, ABC half-transporters and the complicated route to treatment. *Molecular genetics and metabolism,* **90,** 268-276.

Kessel, A., Ben-Tal, N. and May, S. (2001) Interactions of cholesterol with lipid bilayers: the preferred configuration and fluctuations. *Biophysical journal,* **81,** 643-658.

Kim, P. K., Mullen, R. T., Schumann, U. and Lippincott-Schwartz, J. (2006) The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *The Journal of cell biology,* **173,** 521-532.

Kobayashi, S., Tanaka, A. and Fujiki, Y. (2007) Fis1, DLP1, and Pex11p coordinately regulate peroxisome morphogenesis. *Exp Cell Res,* **313,** 1675-1686.

Koch, A., Schneider, G., Luers, G. H. and Schrader, M. (2004) Peroxisome elongation and constriction but not fission can occur independently of dynamin-like protein 1. *Journal of cell science,* **117,** 3995-4006.

Kotti, T. J., Ramirez, D. M., Pfeiffer, B. E., Huber, K. M. and Russell, D. W. (2006) Brain cholesterol turnover required for geranylgeraniol production and learning in

mice. *Proceedings of the National Academy of Sciences of the United States of America,* **103,** 3869-3874.

Kovacs, W. J., Shackelford, J. E., Tape, K. N., Richards, M. J., Faust, P. L., Fliesler, S. J. and Krisans, S. K. (2004) Disturbed cholesterol homeostasis in a peroxisome-deficient PEX2 knockout mouse model. *Molecular and cellular biology,* **24,** 1-13.

Kovacs, W. J., Tape, K. N., Shackelford, J. E., Duan, X., Kasumov, T., Kelleher, J. K., Brunengraber, H. and Krisans, S. K. (2007) Localization of the pre-squalene segment of the isoprenoid biosynthetic pathway in mammalian peroxisomes. *Histochem Cell Biol,* **127,** 273-290.

Laffitte, B. A., Joseph, S. B., Walczak, R., Pei, L., Wilpitz, D. C., Collins, J. L. and Tontonoz, P. (2001) Autoregulation of the human liver X receptor alpha promoter. *Molecular and cellular biology,* **21,** 7558-7568.

Langmade, S. J., Gale, S. E., Frolov, A. et al. (2006) Pregnane X receptor (PXR) activation: a mechanism for neuroprotection in a mouse model of Niemann-Pick C disease. *Proceedings of the National Academy of Sciences of the United States of America,* **103,** 13807-13812.

Lazarow, P. B. and Fujiki, Y. (1985) Biogenesis of peroxisomes. *Annual review of cell biology,* **1,** 489-530.

Li, X., Baumgart, E., Dong, G. X., Morrell, J. C., Jimenez-Sanchez, G., Valle, D., Smith, K. D. and Gould, S. J. (2002) PEX11alpha is required for peroxisome proliferation in response to 4-phenylbutyrate but is dispensable for peroxisome proliferator-activated receptor alpha-mediated peroxisome proliferation. *Molecular and cellular biology,* **22,** 8226-8240.

Lund, E. G., Guileyardo, J. M. and Russell, D. W. (1999) cDNA cloning of cholesterol 24-hydroxylase, a mediator of cholesterol homeostasis in the brain. *Proceedings of the National Academy of Sciences of the United States of America,* **96,** 7238-7243.

Marshall, P. A., Krimkevich, Y. I., Lark, R. H., Dyer, J. M., Veenhuis, M. and Goodman, J. M. (1995) Pmp27 promotes peroxisomal proliferation. *The Journal of cell biology,* **129,** 345-355.

Mauch, D. H., Nagler, K., Schumacher, S., Goritz, C., Muller, E. C., Otto, A. and Pfrieger, F. W. (2001) CNS synaptogenesis promoted by glia-derived cholesterol. *Science (New York, N.Y,* **294,** 1354-1357.

Mitro, N., Vargas, L., Romeo, R., Koder, A. and Saez, E. (2007) T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. *FEBS letters,* **581,** 1721-1726.

Molteni, V., Li, X., Nabakka, J. et al. (2007) N-Acylthiadiazolines, a new class of liver X receptor agonists with selectivity for LXRbeta. *Journal of medicinal chemistry,* **50,** 4255-4259.

Morimura, K., Cheung, C., Ward, J. M., Reddy, J. K. and Gonzalez, F. J. (2006) Differential susceptibility of mice humanized for peroxisome proliferator-activated receptor alpha to Wy-14,643-induced liver tumorigenesis. *Carcinogenesis,* **27,** 1074-1080.

Muse, E. D., Jurevics, H., Toews, A. D., Matsushima, G. K. and Morell, P. (2001) Parameters related to lipid metabolism as markers of myelination in mouse brain. *Journal of neurochemistry,* **76,** 77-86.

Notkola, I. L., Sulkava, R., Pekkanen, J., Erkinjuntti, T., Ehnholm, C., Kivinen, P., Tuomilehto, J. and Nissinen, A. (1998) Serum total cholesterol, apolipoprotein E epsilon 4 allele, and Alzheimer's disease. *Neuroepidemiology,* **17,** 14-20.

Novikoff, P. M. and Novikoff, A. B. (1972) Peroxisomes in absorptive cells of mammalian small intestine. *The Journal of cell biology,* **53,** 532-560.

Olivier, L. M., Kovacs, W., Masuda, K., Keller, G. A. and Krisans, S. K. (2000) Identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes. AA-CoA thiolase, hmg-coa synthase, MPPD, and FPP synthase. *Journal of lipid research,* **41,** 1921-1935.

Pai, G. S., Khan, M., Barbosa, E., Key, L. L., Craver, J. R., Cure, J. K., Betros, R. and Singh, I. (2000) Lovastatin therapy for X-linked adrenoleukodystrophy: clinical and biochemical observations on 12 patients. *Molecular genetics and metabolism,* **69,** 312-322.

Palmer, C. N., Hsu, M. H., Griffin, K. J., Raucy, J. L. and Johnson, E. F. (1998) Peroxisome proliferator activated receptor-alpha expression in human liver. *Molecular pharmacology,* **53,** 14-22.

Peters, J. M., Cheung, C. and Gonzalez, F. J. (2005) Peroxisome proliferator-activated receptor-alpha and liver cancer: where do we stand? *Journal of molecular medicine (Berlin, Germany),* **83,** 774-785.

Poulos, A., Beckman, K., Johnson, D. W., Paton, B. C., Robinson, B. S., Sharp, P., Usher, S. and Singh, H. (1992) Very long-chain fatty acids in peroxisomal disease. *Advances in experimental medicine and biology,* **318,** 331-340.

Reddy, J. K., Rao, M. S., Azarnoff, D. L. and Sell, S. (1979) Mitogenic and carcinogenic effects of a hypolipidemic peroxisome proliferator, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14, 643), in rat and mouse liver. *Cancer research,* **39,** 152-161.

Repa, J. J., Li, H., Frank-Cannon, T. C., Valasek, M. A., Turley, S. D., Tansey, M. G. and Dietschy, J. M. (2007) Liver X receptor activation enhances cholesterol loss from the brain, decreases neuroinflammation, and increases survival of the NPC1 mouse. *J Neurosci,* **27,** 14470-14480.

Riddell, D. R., Zhou, H., Comery, T. A. et al. (2007) The LXR agonist TO901317 selectively lowers hippocampal Abeta42 and improves memory in the Tg2576 mouse model of Alzheimer's disease. *Molecular and cellular neurosciences,* **34,** 621-628.

Rottensteiner, H., Kal, A. J., Hamilton, B., Ruis, H. and Tabak, H. F. (1997) A heterodimer of the Zn2Cys6 transcription factors Pip2p and Oaf1p controls induction of genes encoding peroxisomal proteins in Saccharomyces cerevisiae. *European journal of biochemistry / FEBS,* **247,** 776-783.

Russell, D. W. (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Annual review of biochemistry,* **72,** 137-174.

Schell, M. J., Erneux, C. and Irvine, R. F. (2001) Inositol 1,4,5-trisphosphate 3-kinase A associates with F-actin and dendritic spines via its N terminus. *The Journal of biological chemistry,* **276,** 37537-37546.

Schrader, M., Reuber, B. E., Morrell, J. C., Jimenez-Sanchez, G., Obie, C., Stroh, T. A., Valle, D., Schroer, T. A. and Gould, S. J. (1998) Expression of PEX11beta

mediates peroxisome proliferation in the absence of extracellular stimuli. *The Journal of biological chemistry,* **273,** 29607-29614.

Sengupta, S. and Jordan, V. C. (2008) Selective estrogen modulators as an anticancer tool: mechanisms of efficiency and resistance. *Advances in experimental medicine and biology,* **630,** 206-219.

Shimano, H., Yahagi, N., Amemiya-Kudo, M. et al. (1999) Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *The Journal of biological chemistry,* **274,** 35832-35839.

Sinensky, M. (2000) Functional aspects of polyisoprenoid protein substituents: roles in protein-protein interaction and trafficking. *Biochimica et biophysica acta,* **1529,** 203-209.

South, S. T. and Gould, S. J. (1999) Peroxisome synthesis in the absence of preexisting peroxisomes. *The Journal of cell biology,* **144,** 255-266.

Steffensen, K. R. and Gustafsson, J. A. (2004) Putative metabolic effects of the liver X receptor (LXR). *Diabetes,* **53 Suppl 1,** S36-42.

Steffensen, K. R., Neo, S. Y., Stulnig, T. M., Vega, V. B., Rahman, S. S., Schuster, G. U., Gustafsson, J. A. and Liu, E. T. (2004) Genome-wide expression profiling; a panel of mouse tissues discloses novel biological functions of liver X receptors in adrenals. *Journal of molecular endocrinology,* **33,** 609-622.

Sun, Y., Yao, J., Kim, T. W. and Tall, A. R. (2003) Expression of liver X receptor target genes decreases cellular amyloid beta peptide secretion. *The Journal of biological chemistry,* **278,** 27688-27694.

Tall, A. R. (2008) Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. *Journal of internal medicine,* **263,** 256-273.

Tobin, K. A., Steineger, H. H., Alberti, S., Spydevold, O., Auwerx, J., Gustafsson, J. A. and Nebb, H. I. (2000) Cross-talk between fatty acid and cholesterol metabolism mediated by liver X receptor-alpha. *Molecular endocrinology (Baltimore, Md,* **14,** 741-752.

van der Klei, I. J., Harder, W. and Veenhuis, M. (1991) Methanol metabolism in a peroxisome-deficient mutant of Hansenula polymorpha: a physiological study. *Archives of microbiology,* **156,** 15-23.

van der Zand, A., Braakman, I., Geuze, H. J. and Tabak, H. F. (2006) The return of the peroxisome. *Journal of cell science,* **119,** 989-994.

Wanders, R. J. and Waterham, H. R. (2006) Biochemistry of mammalian peroxisomes revisited. *Annual review of biochemistry,* **75,** 295-332.

Wang, L., Schuster, G. U., Hultenby, K., Zhang, Q., Andersson, S. and Gustafsson, J. A. (2002) Liver X receptors in the central nervous system: from lipid homeostasis to neuronal degeneration. *Proceedings of the National Academy of Sciences of the United States of America,* **99,** 13878-13883.

Wang, Y., Muneton, S., Sjovall, J., Jovanovic, J. N. and Griffiths, W. J. (2008) The Effect of 24S-Hydroxycholesterol on Cholesterol Homeostasis in Neurons: Quantitative Changes to the Cortical Neuron Proteome. *J Proteome Res,* **7,** 1606-1614.

Watkins, R. E., Wisely, G. B., Moore, L. B., Collins, J. L., Lambert, M. H., Williams, S. P., Willson, T. M., Kliewer, S. A. and Redinbo, M. R. (2001) The human nuclear

xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science (New York, N.Y,* **292,** 2329-2333.

Wei, H., Kemp, S., McGuinness, M. C., Moser, A. B. and Smith, K. D. (2000) Pharmacological induction of peroxisomes in peroxisome biogenesis disorders. *Annals of neurology,* **47,** 286-296.

Whitney, K. D., Watson, M. A., Collins, J. L. et al. (2002) Regulation of cholesterol homeostasis by the liver X receptors in the central nervous system. *Molecular endocrinology (Baltimore, Md,* **16,** 1378-1385.

Won, J. S., Im, Y. B., Khan, M., Contreras, M., Singh, A. K. and Singh, I. (2008) Lovastatin inhibits amyloid precursor protein (APP) beta-cleavage through reduction of APP distribution in Lubrol WX extractable low density lipid rafts. *Journal of neurochemistry,* **105,** 1536-1549.

Xie, C., Turley, S. D., Pentchev, P. G. and Dietschy, J. M. (1999) Cholesterol balance and metabolism in mice with loss of function of Niemann-Pick C protein. *The American journal of physiology,* **276,** E336-344.

Yeagle, P. L., Albert, A. D., Boesze-Battaglia, K., Young, J. and Frye, J. (1990) Cholesterol dynamics in membranes. *Biophysical journal,* **57,** 413-424.

Zhang, Y. and Mangelsdorf, D. J. (2002) LuXuRies of lipid homeostasis: the unity of nuclear hormone receptors, transcription regulation, and cholesterol sensing. *Molecular interventions,* **2,** 78-87.

Zhang, Y., Repa, J. J., Gauthier, K. and Mangelsdorf, D. J. (2001) Regulation of lipoprotein lipase by the oxysterol receptors, LXRalpha and LXRbeta. *The Journal of biological chemistry,* **276,** 43018-43024.